VARIATION AND LOCAL ADAPTATION IN STRESS RESPONSE USING STATISTICAL ANALYSIS AND BIOINFORMATICS

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Submitted to the Graduate Faculty of the

Kenneth P. Dietrich School of Arts and Sciences in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy in Ecology and Evolution

University of Pittsburgh

2016

UNIVERSITY OF PITTSBURGH

KENNETH P. DIETRICH SCHOOL OF ARTS AND SCIENCES

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April 1st, 2016

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Across climate regions, organisms have developed various mechanisms that adapt them to their local environments. Understanding the adaptive variation and the underlying genetic basis for the variation can greatly improve our understanding of how natural selection works. Organisms are constantly defending against various environmental stressors. These stressors often share some response pathways, although organisms also deploy stress-specific mechanisms. In this dissertation, I chose to work with natural populations of *Arabidopsis thaliana* across an elevation gradient as a model to explore their variation and local adaptation to various environmental stressors that exist in their local regions. In the first half of my dissertation, I examined the constitutive and induced expression of several key stress response chemicals, camalexin, salicylic acid (SA) and one key heat shock protein (Hsp101), in response to heat stress. Three separate studies were performed using common garden experiments in *Arabidopsis thaliana* seedlings. These chemicals all showed adaptive variation. In the second half of my dissertation, I explored variation and local adaption in response to heat stress, one of the most important stressors in nature, at three different levels. At the phenotypic level, I found out that high elevation plants adopt greater avoidance strategy while low elevation plants adopt greater tolerance to heat stress. At the transcriptomic level, high elevation populations showed more extensive changes in their gene expression in response to heat stress, both in the numbers of differentially expressed genes and the magnitude of the fold change. Finally, at the genomic

level, genome resequencing of 16 populations from our study site also indicated several regions that had undergone selective sweeps, i.e. were genomic targets of strong selection. Summarizing all the studies together, my dissertation provided support of the adaptive divergence in the studied *Arabidopsis thaliana* plants and also provided a framework for exploring other stressors across climate regions.

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PREFACE

I want to start by thanking all the scientists who helped me during my five-year PhD life at the University of Pittsburgh. First, I thank my advisor, Dr. Stephen J. Tonsor. I feel so lucky to have him be my advisor. Our weekly meeting played a significant role in developing my ideas and performing the analysis. Many times, I was amazed by his patience in helping me sort out thoughts when I was stuck and upset about the experiments. He also spent many hours reading my manuscripts and revising them. Every time I talk with him, I feel inspired. I really appreciate all the time and energy he spent in my research and in teaching me how to be a good scientist. I also thank him for maintaining a very relaxing, friendly and open lab community. Our weekly lab meeting provided a great opportunity to get to know the personality and interest of the lab members. The projects and collaborations he has brought in the lab were diverse and interesting. This also inspired me a lot for my research and my personal life. He not only helps me advance my scientific knowledge, but also teaches me many key qualities as a human being. When I get confused and struggled, he is there to help. I am grateful for his support. He makes my PhD life exciting, interesting and motivating. I could not find a better advisor than him.

I also thank the members of my committee. I thank Dr. Nathan Morehouse, who kindly took me into activities in the Morehouse lab and devoted hours for my dissertation and developing my defense presentation in the last year during my PhD life. He was very generous to

share their very new microplate spectrophotometer with me for my research too. He introduced his collaborator, Dr. Nathan Clark at the University of Pittsburgh, for me to consult on bioinformatics related questions. He also gave me very useful suggestions in career development. I thank Dr. Susan Kalisz for her help in my molecular lab work. She was very generous letting me use her centrifuge and PCR machine for a really long time in our lab. She also taught me how to make apple pie. I also want to thank Dr. Tia-Lynn Ashman. Her suggestions on my annual committee meeting were always constructive and innovative. Finally, I thank my outside committee, Dr. Elizabeth Vierling at the University of Massachusetts. She was very supportive for my experiment and always offered suggestions. I especially thank her for the financial support and for helping me select sequencing facilities on the RNA sequencing project.

I also would like to thank my other collaborators. I thank Dr. Brian Traw for his help. He introduced me to the plant hormone field. It was in collaboration with him that I was able to quantify two key plant hormones, salicylic acid (SA) and camalexin, using HPLC. He also trained me to perform RT-PCR. Dr. Joshua Puzey at the College of William and Mary and Dr. John Willis at the Duke University helped me with the whole genome sequencing project. Dr. Willis invited me to Duke University and Dr. Puzey provided me with instructions on library preparation and shared his knowledge on the further sequences analysis. I am grateful to Jennifer Goeckeler-Fried, the lab manager in Dr. Jeffrey Brodsky lab at the University of Pittsburgh. Without her help, I could not have finished quantifying one of the key proteins in my research, heat shock protein 101 (Hsp101) in 600+ samples with Western blots in two months.

I thank the past Tonsor lab members for their support and advice. Natalie Settles, the artist in residence in the Tonsor lab, provided many constructive advices to my experimental design, presentation and poster style. Dr. Tarek Elnaccash introduced me into the world of SAS

and statistics. He was extremely helpful in helping me understand logistical regression analysis and principle component analysis. He helped me perform statistical analysis for my first rotation project. I shared an office with Dr. Marnin Wolfe for two years and I was deeply influenced by his diligence and enthusiasm for plant science. He was also the person who I periodically consulted about lab techniques and greenhouse management during my first two years. I also would like to thank Paul Crawford for his suggestions for presentations. Last, I thank Dr. Alison Hale, who was a postdoc in the Tonsor lab and was my officemate for about a year. Her friendship and company in the office was a great help. I thank the past undergraduate researchers in the Tonsor lab, particularly Brian Belsterling, Jesse Raszewski, Philip Carlucci, Joshua Nguyen, Jai-W Hayes-Jackson. They have directly helped in my research project and were also co-authors for my publications. Without their devotion in time and energy, I could not perform my project in such an effective way. The Tonsor lab managers, Adam Sinder, Kali Theis, and Tim Park, all have helped me in ordering supplies, growing and maintaining plants, and collecting samples.

I would also like to thank the Pitt Ecology and Evolution community. Our Ecology and Evolution community is a small, but really friendly group. I enjoyed our Wednesday seminar, our PEER discussion group, and our annual department retreat at Pymatuning Laboratory of Ecology. In particular, I want to thank Dr. Hao Ji, Dr. Nathan Brower, Dr. Matt Koski, and Mike Chips. During the five years, they have provided me with selfless help, both in my research and in my personal life. I cherish the friendship.

I thank the following sources for financial support. Andrew Mellow Pre-doctoral fellowship, Travel Award from ASPB (American Society of Plant Biology), Graduate Travel Award from ESA (Ecology Society of America), Travel Award from GPSG (Graduate &

Professional Student Government) at the University of Pittsburgh, Tuition & Travel Award from University of Washington, and Arts & Science Mellon Fellowship at the University of Pittsburgh. I also thank Dr. Stephen Tonsor for his support through National Science Foundation.

Last, but not the least, I thank my family. I thank my husband, Lei, for his support. He is the love of my life. Thanks for the encouragement and help. Thanks for always been there for me when I am struggling, confused or depressed. Thanks for sharing the happiness and excitement with me. Thanks for listening me to talk about avoidance and tolerance so many times. Thanks for inspiring me to explore every possibility in my life. Life is so much better after I met you. Our six-month old baby boy, Rieck, is the best. He makes me laugh. He makes me realize my potential and responsibility. He gives me courage to do my best. I appreciate my in-laws for helping us take care of our baby Rieck while I was working on my dissertation. I also thank my parents for their selfless and endless support. I can never thank them enough for their love, support and understanding. I appreciate their courage to support me for pursuing my PhD degree in the United States. This PhD belongs to them, too!

1.0 INTRODUCTION

The planet is never a perfect place for organisms to live. The survival of living organisms depends on their interaction with local environment (Osmond et al. 1987). In fact, all living organisms have to deal with all kinds of stressors around them. Stress is defined as any unfavorable conditions that decrease an organism's ability to grow and reproduce. Stresses cause severe damage in agriculture. Stresses shape species distributions and range expansions. Importantly, stresses from global climate change can act as a modifier of biodiversity and ecosystems. Thus understanding variation and local adaptation in stress response is especially critical to prepare species for the changing global climate conditions.

Fluctuating growing temperature, combined with water limitation, are major factors that limits species' geographic distributions. With a predicted 1.1-6.4 °C global temperature increase by 2020 (IPCC 2007), global climate change will reshape many species' geographic distributions. Some species will reach their known physiological constraints and may eventually become extinct (Root 2003). Importantly, heat and drought can often have combined stress effects, resulting in more severe damage to plants (Hughes 2000). Understanding how plant species vary in their response to current heat stress and their potential to withstand increasing heat stress thus becomes crucial for foreseeing potential species crises as well as for developing effective action for protection. Due to the increasing threat of global climate change, I chose heat

stress as an example to investigate the underlying genetic basis of variation and local adaptation in stress responses in the second half of my dissertation.

Depending on where the stresses come from, stresses can be divided into abiotic and biotic causes. Abiotic stresses are caused by environmental stressors from non-living sources, such as water deficiency, temperature stress, light or nutrient deficiency. Biotic stresses are caused by interactions with living organisms, such as infection by bacteria, virus or fungi, invasive species, or parasitism and predation. Stress responses, abiotic and biotic, share some common pathways, and also have their unique pathways. Organisms are surrounded by these environmental stressors and may eventually evolve adaptive mechanisms that allow them to withstand the stress. These mechanisms include both long- and short- term adaptation. However, every organism has a limited capacity to its stress response and once the limit is exceeded, irreversible damage may happen, leading to cell death (Levitt 1980, Larcher 1987, Lichtenthaler 1996).

All forms of life respond to these various stresses with two strategies: avoidance and tolerance. Stress avoidance is a strategy through which organisms adjust their internal states in ways that reduce exposure to a potentially damaging environment (Touchette et al. 2009, Puijalon et al. 2011). For example, animals can change their behavior, such as running away from the stress, to avoid being exposed to stress; or plants can adjust their morphology to reduce the area being exposed to stress. Stress tolerance is the ability of organisms to minimize or repair damage and maintain functions while experiencing a stress (Touchette et al. 2009, Puijalon et al. 2011). Tolerance allows plants to survive and reproduce in stressful conditions, is very important for organisms to compensate for limited avoidance (Touchette et al. 2009). Stress tolerance includes protection and repair of damaged cell structures, structural proteins, and enzymes (Shah

et al. 2011). Tolerance is mediated by producing stress proteins and chemicals during stress. One important adaptation process is called acclimation. Acclimation occurs in some organisms when pre-exposed to a sub-lethal stress. Stress response at the whole organism level is very complex.

An organism can have very different preference/allocation on avoidance or tolerance depending on the frequency or density of the actual stress in its evolutionary history. For example, lizards are ectothermic and they rely on outside environment to adjust their body temperature. In hot summer, lizards hide in the shade to avoid overheating from the sunshine. However, Alaskan wood frogs tend to freeze into a frog-shaped ice in cold winter, with no breathing and no heart beating. When spring comes, they resume normal activity. This is their way to tolerate cold stress in winter. These fascinating phenomena in nature are not only interesting but also indicate that they have evolved to adapt to their environmental stressors.

Plastic phenotypic variation in response stress often involves an evolved adaptive responses to local climates. Plastic responses result from both gene sequence variation and from variation in expression at the transcriptional level (Lindquist 1986, Swindell et al. 2007, Schoville et al. 2012). Organisms from contrasting climates are thus expected to have different patterns in gene expression. Also, as a result of the long-term local adaptation, populations from extreme climates have evolved differentiation in their genome, and this genomic signature has been revealed in the non-synonymous mutations specific to the extreme conditions, such as hypoxic stress (Gou et al. 2014). A new frontier in ecology and evolution study is to incorporate genomic variation into understanding the phenotypic variation we see in nature across climate regions. Next generation (re-)sequencing (NGS) provides an efficient approach to uncover the underlying genetic basis for complex phenotypic variation (Wang et al. 2009, Qi et al. 2011) . This recently developed method is becoming a powerful molecular tool to understand which

genes are important for stress response in natural populations, and how the molecular stress networks can evolve under diverging environmental stresses. The whole transcriptome expression and whole genome sequences would be the best tools to infer the complex and elaborate genomic expression network variation among and within species during stress response. Currently extensive bioinformatics analysis tools and platforms have been developed to promote usage of NGS data, for RNA-seq (Anders 2010, Haas and Zody 2010, Robinson and Oshlack 2010, Trapnell et al. 2012, Guo et al. 2014), and for whole genome sequencing (Conesa et al. 2005, Librado and Rozas 2009, McKenna et al. 2010, Li 2011).

In plant sciences, measuring stress responses have very often been confined to the study of cellular-level phenotyping or genetic manipulation on particular genes in seedling plants. However, avoidance involves elements of architecture and physiology that are not yet present in seedling plants. Thus many previous studies define their study of stress response as tolerance only. The effect of avoidance in stress response sometimes has been ignored for quite a long time. To the best of my knowledge, currently the genes and pathways involved in stress avoidance are not well understood. The missing knowledge on stress avoidance leaves a huge gap in understanding variation of stress responses and evolution of stress responses, meanwhile also provides great opportunities for me to explore in this dissertation. In addition, the possible trade-offs in avoidance – tolerance strategies are very important because it indicates the constraints and limitations in evolution. However, with studies focusing only on tolerance, we currently have no clear knowledge about the differential allocation of avoidance and tolerance in stress responses, which can provide indications about adaptation to local environmental stressors in evolutionary history. This also provides great challenges in clarifying the variation and local adaptation of avoidance and tolerance strategies in stress responses.

In my research, I am interested to understand a fundamental question in evolution, that is, how do organisms evolve under stress in nature? This is a long-standing question but it is hard to study, because all organisms have various allocations in avoidance and tolerance strategy depending on the details in their local environment. In my dissertation, I first explored phenotypic variation in heat stress, cold stress and bacterial infection of seedlings. Next I focused on heat stress response in adult plants. I explored the variation and local adaptation in heat stress response at three levels: phenotype, transcriptome and genome. In studies on phenotype I characterized the variation in response to stress for plants from contrasting environment. In studies on transcriptome level, I explored the immediate gene expression changes to stress. In studies on genome level, I explored signatures of long-term adaptation to local environmental stressors. Studies at the phenotypic level can tell what variations to stress are there in nature, while studies on transcriptome and genome can indicate how these variations are controlled and how stress responses evolve over time. Overall, I provided a big picture of heat stress response.

In this dissertation, I chose *Arabidopsis thaliana* natural populations in northeast Spain as a model. *Arabidopsis thaliana* is a perfect model for studying plant biology, with short generation time, small genome size and abundant genetic resources. This species is also distributed very broadly across the northern hemisphere, thus we can study its response to many climate conditions. Here the natural populations I chose were from Iberian Peninsula, one of 18 worldwide biodiversity hotspots. In our study region, climate and biotic conditions differ substantially along the elevation gradient. We are therefore able to study adaptation to a range of climate and stress that encompasses much of the climate variation experienced throughout the species' native range in a set of populations apparently derived from common ancestry (Montesinos Navarro et al. 2011).

The sixteen study populations from this region have evolved adaptations to their local climates, especially with regard to temperature and precipitation (Wolfe and Tonsor 2014). The morphological traits measured on the 16 populations showed an cline along elevation from previous studies (Montesinos-Navarro et al. 2009, Montesinos-Navarro et al. 2011, Wolfe and Tonsor 2014). The stressors we imposed in our growth chamber are stressors these populations actually experience in their local environments, but the actual frequency and density may differ among geographic locations. In low elevation sites, plants experience early onset of chronic heat stress and dry soils during the reproductive season. With the onset of chronic stress, plants can either avoid high stress by reproducing early and senescing with the onset of stress or evolve tolerance mechanisms. Plants from higher altitudes and cooler climate are exposed to more variable environments - higher range of temperatures, including higher variability in annual and diurnal temperature. In our high elevation sites precipitation continues longer into the reproductive season (Montesinos et al 2009) so drought stress appears to be less pronounced. In high elevation, plants experience acute and unpredictable heat stress because of the high fluctuation in temperature so high elevation plants are faced with short-term avoidance or tolerance with generally more benign conditions during their reproductive phase. Biotic conditions, such as pathogen pressure, can also shape adaptation in stress response. For example, pathogen density might differ in high elevation compared with low elevation sites because of plant diversity, air pressure, moisture regime, etc. Plants in these environments will experience different mixes of selective pressures and therefore likely evolve different strategies in coping with stresses. My thesis research explored those differences in stress responses.

I firstly explored the phenotypic variation of these populations along an elevation gradient in their response to several abiotic and biotic stressors, including cold, heat stress and

pathogen infection. For the phenotypic variation to various stressors, I adopted a hypothesis-driven approach for performing experiments, and for statistically analyzing the data. These three studies in the first half of my dissertation indicate a general pattern in stress response in these populations, and this pattern is an adaptation in response to local environments. In the second half of my dissertation, I explored the phenotype, transcriptome and genome variation to heat stress in adult *Arabidopsis thaliana* natural plants. Because the seedlings have not yet developed avoidance mechanisms, and because adult plants are much more likely to experience stressfully high temperatures, adult plants were used to study both avoidance and tolerance strategies in heat stress response. To evaluate differential gene expression in response to heat stress and the role of acclimation in altering their gene expression pattern, I adopted the advanced next generation sequencing (NGS) pipeline to obtain transcriptome sequences on eight populations from three treatments, 24 samples total. To explore the population structure and signatures of local adaptation in these populations, I performed whole genome sequencing on 16 populations, 64 genetic lines total. I then performed extensive bioinformatics data analysis from sequence mapping, to identify candidate genes for local adaptation or for stress response, separately. The pipeline developed from the second half of my dissertation can be transferred to study the variation and local adaptation for any other stressors. Below I provided a more detailed description of the experiments and key results from each chapter of my dissertation.

Chapter 2, 3, and 4, the first half in my dissertation, explored the phenotypic variation in these populations with or without stress in seedlings. Seedlings are commonly used in such studies to explore specific cellular mechanisms.

In Chapter 2, I show a geographic cline in leaf salicylic acid (SA) with increasing elevation in these populations. SA is a key plant hormone, in response of both abiotic stress and

pathogen infection. Here I provide evidence of local adaptation. Overall, SA level decreased significantly in response to a 44^{o}C heat treatment. I further showed that the elevational cline in leaf SA level might be related with the increasing pathogen pressure at higher elevation. High elevation might experience more pathogens. This work was done in collaborating with Dr. Stephen Tonsor and Dr. Milton Brian Traw at the University of Pittsburgh and is published in *Plant Signaling & Behavior* (Zhang et al. 2015b).

In Chapter 3, I focus on another key plant hormone, camalexin, an important defense chemical against bacterial or fungal pathogens. This chapter intends to test whether camalexin expression variation supports the optimal defense theory, that induction of defensive secondary metabolites in plants is inversely correlated with constitutive expression of those compounds, tested by using genetic lines collected in study sites at four elevations. Induction of camalexin, when plants were exposed to 48hr of a laboratory standard pathogenic bacterial line, DC3000, was negatively related with constitutive expression of camalexin. However, constitutive overexpression in high elevation genetic lines was not explained by the only known natural genetic effector of camalexin expression, a polymorphism at the Accelerated Cell Death 6 (ACD6) gene. This work was done in collaboration with Andy Lariviere, Dr. Stephen Tonsor and Dr. Milton Brian Traw at the University of Pittsburgh. This work is published in *Plant Science* (Zhang et al. 2014).

In Chapter 4, I report a study of heat stress tolerance (that is, thermotolerance) variation among populations. I ask whether one heat shock protein, Hsp101, can account for the variation we observed. Heat shock proteins (Hsps) are rapidly up-regulated in heat stress and Hsp101 is the only Hsp that has shown to be necessary for acquiring thermotolerance in *Arabidopsis thaliana*. I show how the extent of Hsp101 expression and thermotolerance are interplayed; both

appear to evolve adaptively in natural populations. This work was conducted with Dr. Stephen Tonsor and two undergraduate researchers, Brian Belsterling and Jesse Raszewski, at the University of Pittsburgh. This work is published in *AoB PLANTS* (Zhang et al. 2015a).

Starting from Chapter 5, the second half of the dissertation, I concentrate on heat stress in adult plants, one of the most important stresses in nature, with increasing importance due to global climate change. I use heat stress as an example to explore the comprehensive stress response, including avoidance and tolerance in adult plants. In seedlings, the capacity for avoidance has not yet developed because it relies on structures and behaviors that are not themselves fully developed in seedlings. It is in adult plants that we start to see a big picture on the complex and adaptive avoidance – tolerance strategies contributing to heat stress response.

In Chapter 5, I show contrasting avoidance – tolerance strategies in plants from low and high elevation plants when repeatedly exposed to a 45^{o}C heat stress for 3hrs. High elevation plants adopt greater avoidance and low elevation plants adopt greater tolerance. The specific mechanisms for each of avoidance and tolerance are also explored. This work was conducted in collaboration with Dr. Stephen Tonsor and three undergraduate researchers, Philip Carlucci, Joshua Nguyen and Jai-W Hayes-Jackson, at the University of Pittsburgh. This work is currently under review in *Ecology* (Zhang et al. under review) and is published on bioRxiv for preprint (bioRxiv doi: http://dx.doi.org/10.1101/044461) .

In Chapter 6, I focus on identifying significantly differentially expressed genes in heat stress response and the role of acclimation for populations from low and high elevations using 24 RNA sequencing samples. I show low elevation plants adopted more small Hsps but high elevation plants adopted more large Hsps in gene expression. Low and high elevation plants also differ in the genes involved in reactive oxygen species (ROS). The contrasting patterns in Hsps

and ROS genes indicate low and high elevation plants have evolved divergent patterns in gene expression to high temperature stress, both pre-acclimation and direct exposure. This work was conducted in collaboration with Dr. Elizabeth Vierling at the University of Massachusetts, and Dr. Stephen Tonsor at the University of Pittsburgh. The work is currently published on bioRxiv, for preprint (bioRxiv doi: http://dx.doi.org/10.1101/044446) and will be submitted for peer review soon.

In Chapter 7, I explore the genomic variation in 16 populations and look for regions that show evidence of strong selection. By so doing, I hope to elucidate the underlying genetic basis of adaptation to low and high elevation. Whole genome sequence data support the hypothesis that these populations can be classified in three elevation groups: very high, high & middle, and low. I also explore genes involved in adaptation to very high elevation and adaption to low elevation, respectively. This work was done in collaboration with Dr. Joshua Puzey now at College of William and Mary, Dr. John Willis at Duke University, and Dr. Stephen Tonsor at the University of Pittsburgh.

In Chapter 8, I conclude by summarizing my results and discussing their relevance and importance in ecological evolution and in the context of global climate change. I also outline topics for future study in stress response.

Our extensive exploration on the study system has provided substantial advances in understanding of the population level adaptive response to various stressors. The dissertation provides a framework for studying complex stress responses in any stressors with statistical analysis and bioinformatics tools. The framework is transferable and the output from this study sheds lights on stress response in general.

2.0 A GEOGRAPHIC CLINE IN LEAF SALICYLIC ACID WITH INCREASING ELEVATION IN *ARABIDOPSIS THALIANA*

2.1 INTRODUCTION

At a broad geographic scale, plant assemblages can be predicted largely by differences in annual temperature and precipitation (Whittaker 1970). Plants within a climatic region converge evolutionarily on particular morphological, physiological, and phenological themes, which are particularly notable in association with the increased risk of cold stress with increasing latitude or elevation (Warming and Vahl 1909, Clausen and Hiesey 1958). While hormones regulate much of the variety that we see in plant morphology and physiology, the extent of variation in the production of plant hormones in relation to latitudinal and elevational clines remains largely unknown (Traw and Bergelson 2010).

High elevation environments present significant challenges from both abiotic and biotic stress. Notable abiotic stresses include the increased exposure to ultraviolet radiation (UV) and increased frequency of freezing temperatures(Körner 2003). It is not surprising therefore that the abundance and diversity of most taxonomic groups are lower in high elevation habitats (Burdon et al. 1992, Körner 2003, Bryant et al. 2008, Reymond et al. 2013). While the diversity of pathogens is likely to be lower at high elevations (Bryant et al. 2008), their overall impact on hosts could exceed those at low elevation for several reasons. Because plant diversity is low in

these habitats (Körner 2003), outbreaks of pathogens are transmitted more readily among hosts (Burdon et al. 1992, Barbeito et al. 2013). Because predators are less abundant in these habitats (Reymond et al. 2013), there is less potential for top-down control of outbreaking pests (Haukioja 2005). Finally, the virulence of some plant pathogens such as snow blight fungi (Burdon et al. 1992, Barbeito et al. 2013) and ice nucleating bacteria (Morris et al. 2008) is facilitated by cold conditions.

Plants at high elevations have been shown to exhibit elevated constitutive concentrations of phenolic compounds, particularly flavonoids and phenolic glycosides (Alonso-Amelot et al. 2004, Zidorn et al. 2005, Alonso-Amelot et al. 2007, Rieger et al. 2008, Spitaler et al. 2008, Xenophontos et al. 2008). These compounds consist of aromatic rings which absorb UV radiation and in some cases have been shown to reduce the deleterious effects of oxidative damage to plant tissues (Li et al. 1993). Some of these compounds have been shown to increase in concentrations in plants following treatment with cold temperatures (Bilger et al. 2007, Albert et al. 2009) and have been correlated recently with high antibiotic activity against pathogenic bacteria (Martz et al. 2009). However, the hormonal dynamics underpinning these elevational differences in phenolic chemistry have not been identified previously.

Perhaps the most widely represented phenolic in plants is the major plant hormone, salicylic acid (SA). SA has a central role in orchestrating the cascade of plant induced defenses against bacterial pathogens and some insect and fungal pests (Vlot et al. 2009). One of the important defense compounds against bacterial and fungal infection is camalexin, 3-thazole-2-yl-indole, which, like SA, is also derived from chorismate (Vlot et al. 2009). Interestingly, SA also has notable importance in thermal regulation, owing to its involvement in decoupling electron transport in mitochondria and the release of energy in the form of heat (Raskin et al. 1989). SA

has particularly strong effects on thermal regulation in the arum family, Araceae, (Raskin et al. 1989), but cold temperatures have been shown to induce accumulation of SA also in the model plant, *Arabidopsis thaliana* (Leyva et al. 1995, Kaplan et al. 2004). On the basis of these studies, a recent review (Traw and Bergelson 2010) predicted that SA concentrations would likely be higher in *A. thaliana* at high elevations.

In the current study, we asked specifically whether SA concentrations increase with increasing elevation across a key region of endemism of *A. thaliana* on the Iberian Peninsula (Wolfe and Tonsor 2014) and how these concentrations relate to particular aspects of climatic variation. If leaf constitutive SA concentrations were found to be higher at high elevations, then that would be consistent with an important role of the abiotic or biotic stresses associated with the high elevation habitats. We then asked how a high temperature treatment would alter leaf concentrations of SA and finally whether leaf camalexin concentrations also varied across the elevational gradient.

2.2 MATERIALS AND METHODS

2.2.1 *Arabidopsis* material and growth conditions

Characteristics of the fifteen source populations on the Iberian Peninsula (Fig. 1A, Table 14) have been described extensively elsewhere (Zhang et al. 2014) and seed stocks are publically available at the Arabidopsis Biological Resource Center of Ohio State University (ABRC CS#78884). The first climate principle component (Climate PC1) explains 71% of climate

variation across the elevation gradient associated with the source populations (Wolfe and Tonsor 2014) (Fig. 1B, Table 14).

To assess leaf concentrations of SA across the elevational gradient, we first performed a common garden experiment in 2009 using seeds from eight source populations across the elevation gradient and including nine genotypes from each population. For four of the populations (ARB, POB, MUR, and VDB), we measured one replicate plant per genotype for SA for a total of 36 plants. For four of the populations (BAR, HOR, ALE, and VIE), we had more available seeds and for these populations we had two replicate plants per genotype for constitutive measurement of SA, for a total of 72 plants and a cumulative total of 108 plants (Table 15). We surface sterilized seeds and then planted them onto 36-cell flats filled with Promix-BX potting soil and placed them for one week in a 4ºC cold room for cold stratification. We then transferred the flats to a growth room at the University of Pittsburgh with a constant temperature of 20ºC and a 14hr day length with light levels of 350μmol m^{-2} sec^{-1} provided by a 1:1 mixture of sodium and metal halide lamps. At seven days past germination, we thinned to three seedlings per cell and at ten days past germination, we thinned to one seedling per cell. We then subdivided the cells and assigned each plant to a specific treatment and then completely randomized and redistributed the cells across the growth room bench. Cells were moved at least once per week within the growth chamber to reduce positional effects.

To assess the direct effects of a heat treatment on SA concentrations, we also grew an additional two replicates per genotype for the same four populations (BAR, HOR, ALE, and VIE) for an additional 72 plants that were randomized along with the other replicates, but exposed to a 44ºC treatment in the dark for 3 hrs beginning on Day 25. Control plants also placed in the dark for 3 hr but were kept at the ambient temperature of 20 ºC. We then re-

randomized the heat treatment and control plants together in the growth room and harvested all 180 plants on Day 30 of growth when all plants had rosettes and bolting had not yet been initiated.

To reassess SA concentrations across the elevational gradient and to measure leaf camalexin concentrations, we conducted an additional common garden experiment in 2013 using seeds from the larger set of 15 populations. For these measurements, we included one replicate of each of four genotypes from each of these 15 populations, for a total of 60 plants. Seeds were cold stratified at 4ºC for five days and then grown at 22°C for 3 weeks (16hr light/8hr dark) in growth chambers. Rosettes were then exposed to a four-week vernalization at 4ºC to synchronize flowering time followed by 22°C for three weeks (16hr light/8hr dark) at which point they began flowering. Plants were randomly distributed in the growth chamber and were rotated every two days to minimize within-chamber effects. Leaf materials for the measurement of SA were collected at the start of flowering (Table 16).

2.2.2 Measurement of leaf SA and camalexin concentration

To measure leaf SA and camalexin concentrations we first harvested rosettes, froze them in liquid nitrogen, and stored them at -80ºC prior to analysis. In the 2009 experiment, we extracted directly from the frozen tissue, and calculated SA concentrations on a wet mass basis, whereas in the 2013 experiment we lyophilized the tissue to dryness first and then calculated concentrations on a dry mass basis (µg/g leaf dry mass). To control for run to run variation, we added an internal control of 1µg of O-Anisic Acid (Sigma # 169978) consisting of 100µl from a stock solution of 10µg of O-Anisic Acid in 1ml of 100% methanol. To extract SA, we weighed leaf tissues, suspended them in 3ml of 90% methanol, rocked the tubes in a shaker at room

temperature for 24hr, and transferred the supernatant to a new tube. We then resuspended the pellet in 3ml of 100% methanol, vortexed, rocked the tubes again for 24hr, and combined the two supernatant fractions. To capture both the free SA and the SA conjugated to sugar, we then split each sample into two equal volumes into two screwcap tubes and placed the tubes in a fume hood until dry, which required at least 48hr. The first aliquot was used to measure free SA, and the second was used to measure total SA, which includes both the free and sugar-conjugated SA. To cleave the sugar from the SA glucoside, we added 40U of b-glucosidase enzyme (Sigma # 0395) in 400µl of 100mM sodium acetate buffer (pH 5.5) to the total SA aliquots. The paired free SA aliquot received 400µl of buffer only. All tubes incubated overnight at 37^{o}C. To deactivate the enzyme, we then added 400µl of 10% trichloroacetic acid to all samples. To partition the SA from other compounds, we then extracted each tube twice with 1ml of an organic solvent mixture (100:99:1 of ethyl acetate: cyclopentane: 2-propanol) and vortexed. We then collected the two organic phase fractions in a centrifuge tube, which we then placed in a fume hood until dry, which required at least 24 hrs. We resuspended the organic fraction in 600µl of 55% methanol, vortexed, and rocked overnight. To filter out impurities, we passed the supernatant through 0.2µm nylon spin-prep membrane filters (Fisher #07-200-389). To measure SA concentrations by high performance liquid chromatography (HPLC), we used an HP1100 system with a 4.6 x 150mm Agilent Eclipse XDB C-18 column and fluorescence detector, with excitation at 301nm and emission at 412nm for SA, excitation at 301nm and emission at 386nm for camalexin, excitation at 301nm and emission at 365nm for O-anisic acid. The flow rate was 1 ml/min and the solvents were A) 100% methanol and B) 0.5% acetic acid in water. Each run consisted of 30% A and 70% B for the first five minutes, increasing to 40% A at 7.5min and 60% A at 15min, returning to 30% A at 18min. To calculate concentrations of free SA, total SA,

and camalexin, we divided the peak area of each compound by the product of the peak area of the O-Anisic acid internal standard and the sample mass in grams.

2.3 RESULTS

To assess the hypothesis that SA concentrations increase with increasing elevation across a key region of endemism of *A. thaliana* on the Iberian Peninsula (Fig. 1A), we conducted two large common garden experiments which allowed us to test the genotypes from the different source populations together under a common set of environmental conditions, and found that SA concentrations declined significantly with increasing elevation in both experiments, contrary to the predictions. In the larger experiment conducted in 2013 and including 15 source populations, we found that genotypes from the high elevation source populations produced an order of magnitude less free SA per dry mass relative to populations from lower elevations (R^2 = 38.6%, P=0.013, Fig.2A). There was also a significant decline in total SA per dry mass with increasing elevation (R^2 = 24.7%, P=0.059, Fig.A1A). In the 2009 common garden experiment conducted on the smaller set of eight populations, we found that genotypes from the low elevation source populations did not differ significantly in free SA (R^2 = 30.9%, P=0.15, Fig. A1B), but produced roughly two-fold more total SA per wet mass relative to the populations from the higher elevations (R^2 = 69.9%, P=0.010, Fig. A1E).

Free SA per dry mass was strongly negatively correlated with climate PC1 in the 2013 experiment (R^2 = 27.9%, P=0.043, Fig.2B) where higher values of PC1 corresponded to colder and wetter habitats. The climate PC2 axis did not explain significant variation in leaf free SA and did not improve the fit of the model when included with climate PC1 (Table 17). Total SA

was not correlated significantly with climate PC1 (R^2 = 15.9%, P=0.14, Fig. A1C) or climate PC2 (R^2 = 0.12%, P=0.90). In the 2009 experiment, free SA was not correlated with climate PC1 (R^2 = 31.3%, P=0.14, Fig. A1D), but total SA concentration was correlated significantly with climate PC1 (R^2 = 67.1%, P=0.013, Fig. A1F).

To assess whether a heat treatment itself could induce changes in SA concentrations, we also challenged plants from four populations (BAR, HOR, ALE, and VIE) along the elevation gradient with exposure to 44^{o}C for 3 hr. Leaf free and total SA concentrations decreased by 10.8% and 11.2%, respectively in the heat treated plants relative to the control plants ($F_{1,64}$=6.3, P=0.015, Fig. 3A and $F_{1,64}$=5.1, P=0.026, Fig. A2, respectively) and these responses to the heat treatment did not differ significantly among the four populations, as indicated by the non-significant population by treatment interaction term in the analysis of variance.

To determine whether levels of the anti-bacterial defenses also changed across the elevation gradient, we then assessed leaf camalexin concentrations in the 11 populations that had non-zero concentrations of this compound in the 2013 experiment. Interestingly, the four populations lacking camalexin production (PIN, RAB, BAR, and HOR) were all located at low elevations. For the eleven populations that had non-zero levels of camalexin production, we found that leaf constitutive concentrations of camalexin declined significantly with increasing elevation (R^2 = 52.8%, P=0.011, Fig. 3B, Table 19).

2.4 DISCUSSION

SA is perhaps the most widely distributed phenolic compound in plants and is central to defense against bacterial pathogens (Vlot et al. 2009). A recent review (Traw and Bergelson 2010) predicted that SA concentrations would likely be higher in plants at high elevations, given evidence that cold temperatures directly stimulate SA production in laboratory plants (Leyva et al. 1995, Kaplan et al. 2004) and that concentrations of phenolics such as flavonoids and phenolic glycosides are typically very high in leaves of high elevation plants (Alonso-Amelot et al. 2004, Zidorn et al. 2005, Alonso-Amelot et al. 2007, Rieger et al. 2008, Spitaler et al. 2008, Xenophontos et al. 2008, Albert et al. 2009, Martz et al. 2009). We report here that SA concentrations in *Arabidopsis thaliana* from wild populations on the Iberian Peninsula do not support the predicted pattern. Indeed, the cline of SA was in the opposite direction from what we expected, with concentrations higher on average in low elevation populations. These findings are robust in that they were observed in two separate common garden experiments, conducted using both frozen and lyophilized leaf tissues. To our knowledge, this is the first evidence of a geographical cline in SA concentrations in plants.

There are several possible explanations for the unexpected pattern of a decrease in SA with increasing elevation observed here. First, warm temperatures might directly stimulate plant production of SA. Many studies have shown consistently that warm temperatures actually suppress SA concentrations in *A. thaliana* and other plants [reviewed in (Traw and Bergelson 2010). To assess the possibility that these wild Spanish populations exhibited a different pattern, we directly exposed plants of four of the Spanish populations to an elevated temperature treatment of 44^{o}C for 3 hr and found that the high temperature treatment reduced SA concentrations (Fig. 3A), in agreement with the general pattern in the literature (Yang and Hua

2004). Thus, the high SA concentrations that we observe in low elevation plants cannot be explained directly by the warmer temperatures found at low elevations. However, the ability of plants to tolerate heat stress may require high levels of SA, as has been suggested in studies of acquired high temperature tolerance in laboratory experiments with seedlings of *A. thaliana* (Larkindale et al. 2005). To our knowledge, no experimental work has examined the relationship of constitutive SA expression levels in wild plants to their survival under high temperature stress. Whether heat stress survival may explain the climate/elevation cline in SA observed here remains to be explored.

A second possible explanation for why SA concentrations would decrease with increasing elevation relates to source-sink dynamics. All phenolic compounds, ranging from simple compounds such as SA to complex polyphenolics, are derived from a common resource pool of chorismate (Chen et al. 2009). If the complex polyphenolics have greater efficacy in protecting plants from UV stress, then plants at high elevations may shunt more of the chorismate toward this sink and away from the production of SA. There is currently insufficient data to address this hypothesis. However, we did measure the concentrations of camalexin, which is another chorismate-derived compound (Mao et al. 2011) and found that camalexin concentrations were also lower at high elevations (Fig. 3B), which would not support a source-sink explanation.

A third possible explanation for our pattern of higher SA levels in low elevation populations relates to the likelihood that overall enemy pressures are likely to be greater in low elevation environments. Under this scenario, plants at the lower elevations have evolved higher constitutive levels of SA as a consequence of strong selection by enemies. Bacterial diversity and abundance is likely to be higher at low elevations (Bryant et al. 2008). Furthermore, recent

studies have found that pathogen loads on plants are higher at lower elevations (Springer 2007) and resistance to enemies is lower at high elevations (Pellissier et al. 2014). It is possible that higher SA concentrations are needed in plants at low elevations to combat pathogens in those habitats, but at this point, manipulative experiments are needed to assess the relative impacts of these key environmental variables.

Concentrations of SA for *A. thaliana* previously reported in the literature have been typically around 1µg/g wet mass of leaf tissue, which is lower than the average value for our data of 4.7µg/g wet mass for free SA from the 2009 experiment (95% confidence interval of 4.4 to 5.0, Table 15). We have carefully checked and rechecked our calculations and methodology and see no explanation for this difference. We are confident that the elevational pattern is robust, despite this lack of congruence. These natural Spanish lines may have constitutively higher SA concentrations than have been described previously in the literature; however, future studies should include the Col-0 and Ws-0 lines alongside the laboratory lines for better calibration of our findings relative to those of other labs.

In other work, we have found that SA and camalexin concentrations do explain some variation in plant resistance to attack by a bacterial pathogen (Zhang et al. 2014). Thus, our finding of a geographic cline in SA and camalexin concentrations may contribute to understanding how plant resistance to bacterial infection is distributed in natural populations. Geographic clines in plant compounds, such as those examined here, are likely to provide information that will be important for understanding how plant populations will be affected by a warming climate (Boland et al. 2004).

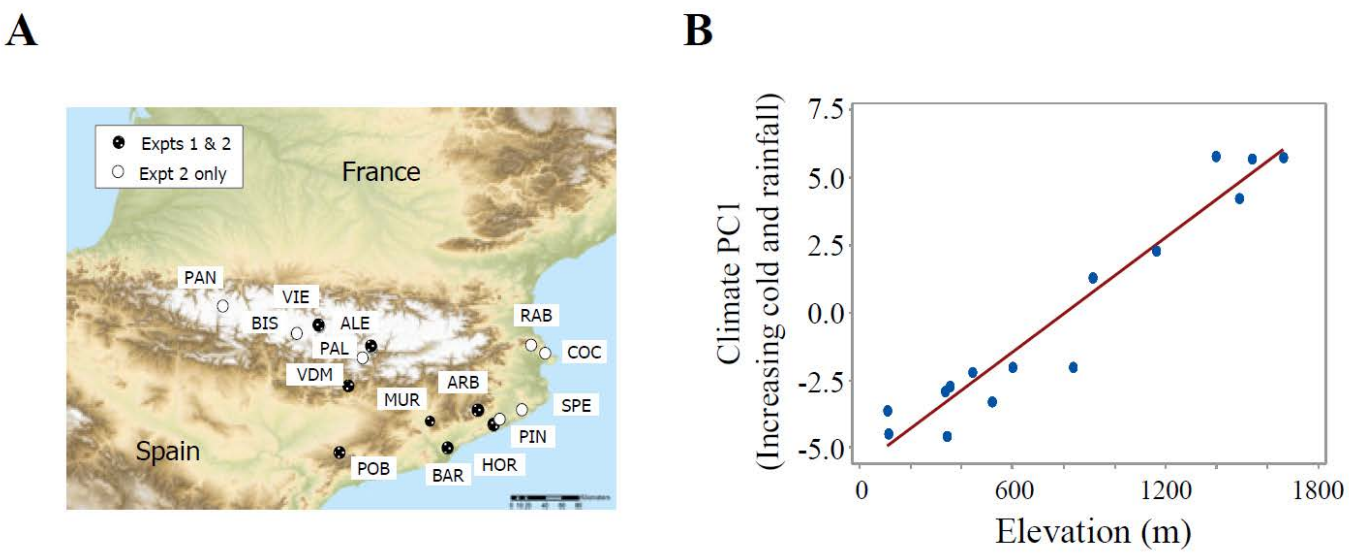


Figure 1. Collection of fifteen populations of *A. thaliana* across an elevational transect on the Iberian peninsula [as shown in (Wolfe and Tonsor 2014) showing A) populations used in each of the two experiments and B) the relationship between climate PC1 and elevation.

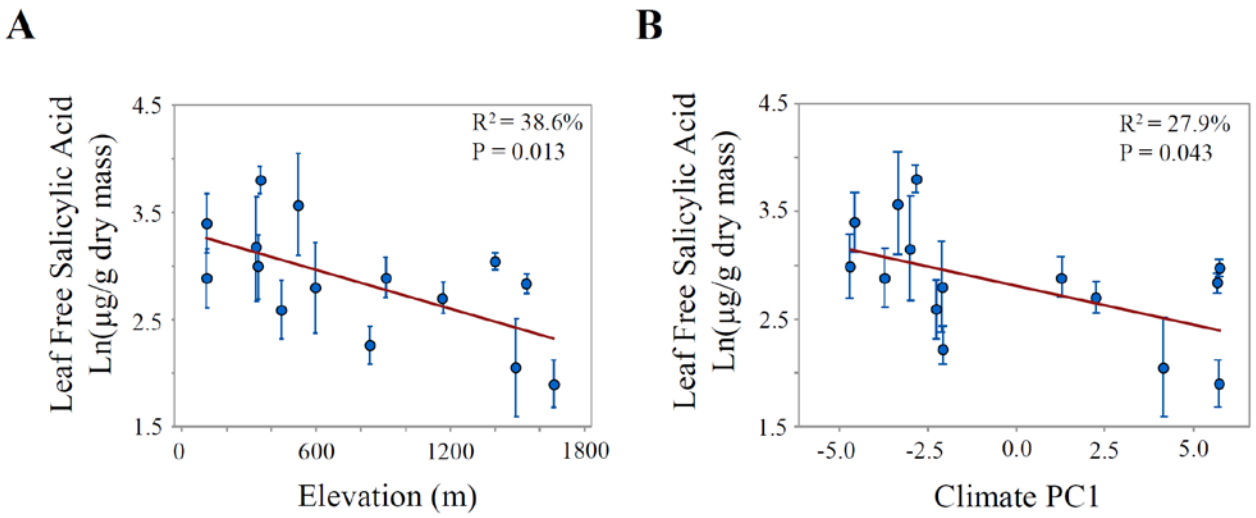


Figure 2. Regressions of population means from the 2013 common garden experiment showing relationships between leaf free salicylic acid concentration and A) elevation in meters and B) climate PC1, where higher values represent colder temperatures and greater rainfall. Shown are population means (+/- 1SE) for ten-week-old plants representing four maternal families per population.

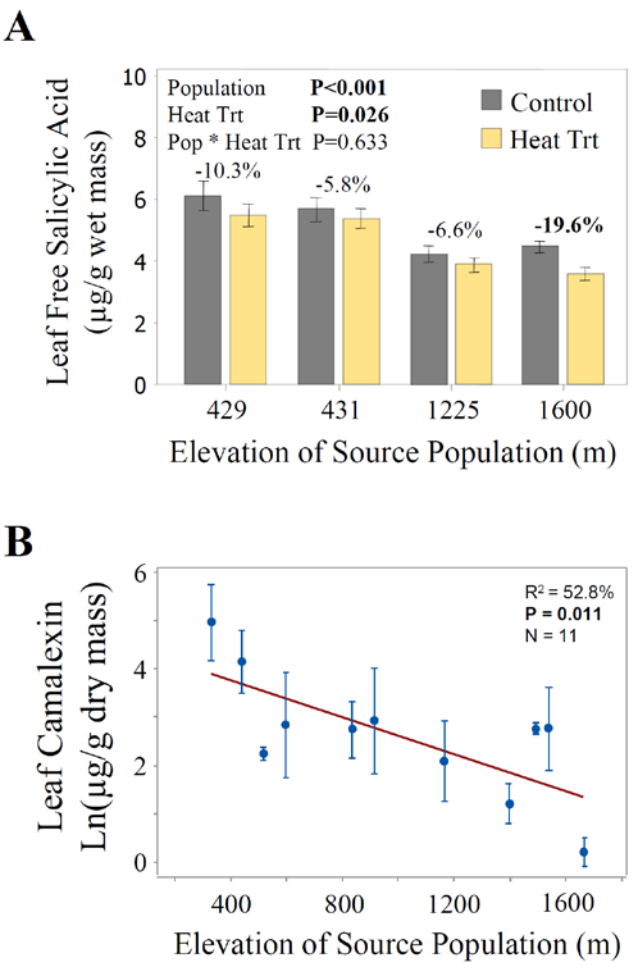


Figure 3. A) Effect of a heat treatment of 44ºC for 3 hr on leaf concentrations of free SA in plants from four source populations: BAR (429m), HOR (431m), ALE (1225m), and VIE (1600m). Each population was represented by eight or nine independent maternal lines, each represented by two replicates in each of the two treatments, for a total of 144 plants (Table 18). B) Regression of population means of natural log transformed leaf camalexin concentrations from 11 populations in the 2013 common garden experiment as a function of the elevation of the source population in meters (Table 19). Four populations (PIN, RAB, BAR, and HOR) lacked constitutive leaf camalexin concentrations and were excluded from the regression. Significant P values are indicated in bold.

3.0 CONSTITUTIVE CAMALEXIN PRODUCTION AND ENVIRONMENTAL STRESS RESPONSE VARIATION IN *ARABIDOPSIS* POPULATIONS FROM THE IBERIAN PENINSULA

3.1 INTRODUCTION

Because of their stationary existence, plants experience a very broad range of environmental stresses ranging from large diurnal and seasonal temperature fluctuations to attack by herbivores and pathogens. Plants respond to these environmental stresses with complex sets of defenses that are coordinated in part by the activity of three important hormones: jasmonic acid, abscisic acid, and salicylic acid (SA) (Vlot et al. 2009). Much of what is known about these hormones has come from the study of agricultural crops (Vlot et al. 2009, Hua 2013). How these hormones are expressed and coordinated with defense in natural populations remains largely unknown and is an important area of current study (Traw and Bergelson 2010).

Plant allocation to a chemical defense can be partitioned into a constitutive level and an induction response (Morris et al. 2006). The constitutive level is the amount of the defense that plants express under normal environmental conditions, whereas the induction response is the amount of additional allocation to defense produced in response to an environmental signal associated with a greater need for defense. Optimal defense theory (McKey 1974) predicts that populations that experience more attack would express greater constitutive allocation to defense,

whereas populations that face infrequent attack are predicted to have lower constitutive levels, owing to the associated costs, but greater allocation to induced expression(Zangerl and Bazzaz 1992). As a consequence, the theory predicts that induction responses is negatively correlated with constitutive concentrations of these same plant defenses. Despite a number of studies that have assessed the relationship between constitutive and induced resistance (reviewed in Morris et al. 2006), the relationship remains unclear. These relationships have been challenging to address because the genetic basis of constitutive variation in defense among genotypes has not been identified.

SA is critical for plant resistance to pathogens (Vlot et al. 2009) and has been shown previously to increase in response to cold temperatures in *Arabidopsis thaliana* (Leyva et al. 1995, Kaplan et al. 2004) and to regulate thermal responses in the family Araceae (Raskin et al. 1989). SA levels increase strongly in response to damage by bacterial pathogens (Vlot et al. 2009). In some plants, including *Arabidopsis thaliana*, the downstream responses to bacterial infection also include production of additional aromatic compounds, such as phytoalexins, which provide defense for plants infected by pathogenic fungi and bacteria.

Camalexin, 3-thiazole-2-yl-indole, is one of the principle phytoalexins produced in *Arabidopsis thaliana* (Tsuji et al. 1992, Zook and Hammerschmidt 1997, Ren et al. 2008). Camalexin accumulation is known to inhibit the growth of virulent strains of *Pseudomonas syringae* (Tsuji et al. 1992). Exogenous purified camalexin also inhibited the growth of a fungus, *Botrytis cinerea,* in a dose-dependent manner (Ferrari et al. 2003). Because camalexin is found typically in plants under attack by pathogens, it is often used as an indicator of plant pathogen-induced stress. To date, there is only one well-documented example of constitutive production of camalexin in natural populations (Todesco et al. 2014). In that case, some wild lines of

Arabidopsis thaliana possess a hyperactive allele of the Accelerated Cell Death 6 (ACD6) gene, which triggers immune system responses even in the absence of enemies. ACD6 encodes an ankyrin-repeat protein that is an important component of plant resistance to bacterial infection (Rate et al. 1999, Lu et al. 2003). Little is currently known about the geographic distribution of the natural alleles of this gene (Todesco et al. 2014).

Arabidopsis thaliana, hereafter "*Arabidopsis*," provides a particularly strong system for addressing the role of secondary compounds in plant tolerance of environmental stress, owing to the primary representation of the single phytoalexin, camalexin, and the extensive worldwide sampling of genotypes of this species across elevational gradients. It also has well-established interactions with pathogenic bacteria in the genus *Pseudomonas*, including a number of wild strains that are collectively identified as *Pseudomonas syringae* (Jakob et al. 2002, Traw et al. 2007). Because *Pseudomonas* bacteria travel throughout the global hydrological cycle, including as the nuclei of snowflakes, they disperse across the full latitudinal and elevational range of plant populations(Morris et al. 2008).

Here, we conducted three experiments addressing relationships between constitutive and induced levels of camalexin in natural populations of *Arabidopsis thaliana* from the Iberian Peninsula. In the first experiment, we studied leaf camalexin response to a cold treatment. In the second and third experiments, we examined the effects of high constitutive concentrations of camalexin on bacterial growth and the induction of camalexin following bacterial infection, respectively. Specifically, we asked the following four questions: (1) Do leaf concentrations of camalexin increase following plant exposure to a cold treatment? (2) Do the high constitutive leaf camalexin levels present in the VIE population correlate with decreased growth of the virulent bacterial pathogen, *Pseudomonas syringae* pv. tomato DC3000? (3) Do genotypes with

the highest constitutive concentrations of camalexin have the lowest induction responses, as predicted by optimal defense theory? (4) Does natural genetic variation at the ACD6 locus [14] explain the observed variation among genotypes in leaf constitutive camalexin concentration?

3.2 MATERIALS AND METHODS

3.2.1 *Arabidopsis* material and growth conditions

We focus on a collection of four *Arabidopsis* populations on the Iberian Peninsula that have been characterized extensively elsewhere (Pico et al. 2008, Gomaa et al. 2011, Montesinos-Navarro et al. 2011, Montesinos-Navarro et al. 2012). Seeds were originally collected from the Northeastern Iberian Peninsula in Spain (Fig. 31) and subsequently propagated by single seed descent for three generations in the Tonsor lab. These stocks are publically available at the Arabidopsis Biological Resource Center of Ohio State University (ABRC CS#78884). Genomic analyses show that these *Arabidopsis* populations have a history of genetic isolation from populations elsewhere in the Iberian Peninsula (Pico et al. 2008, Montesinos-Navarro et al. 2011).

Four structured populations, each containing eight or nine maternal lines, were chosen for this study. The climate characteristics of these four populations are shown in Table 1. Seeds were surface sterilized and planted with 10-15 seeds per cell onto 36-cell flats filled with Promix-BX potting soil. Flats were placed in a 4^{o}C cold room for 1wk of cold stratification. Flats were then moved from the cold room to a large walk- in environmentally controlled room at the University of Pittsburgh with a constant temperature of 20^{o}C and a 14hr day length with

light levels of 350μmol m^{-2} sec^{-1} provided by a 1:1 mixture of sodium and metal halide lamps. Flats were thinned to three seedlings per cell at seven days past germination. Flats were then subdivided and each cell was assigned to a specific treatment and then completely randomized and redistributed across the growth room bench. Plants were moved at least once per week within the growth chamber to reduce positional effects.

3.2.2 *Pseudomonas syringae pv. Tomato* DC3000 growth conditions

Pseudomonas syringae pv. tomato DC3000 (hereafter referred to as "Pst DC3000") is a primary model bacterial pathogen for use with A. thaliana (Hirano and Upper 2000). To generate the inoculation solution, we streaked bacteria on King's B medium (King et al. 1954) containing 50ug/l rifampycin and transferred a single colony to 50ml of liquid media with 50ug/g rifampycin, which was shaken in an incubator for 24hr at 28oC (Traw et al. 2003). An aliquot of 5ml was then transferred to a fresh 45ml of liquid media with 50ug/g rifampycin and was shaken in an incubator for 4hr at 28oC to achieve mid-logarithmic stage growth, and then spun for 5min at 3000rpm to obtain a pellet. The pellet was resuspended in 1ml of 10mM MgSO4, vortexed, measured for bacterial concentration by spectrophotometry and diluted to the inoculation concentration of 1x105 cfu/ml.

3.2.3 Experimental treatments

In the first experiment, we asked whether the *Arabidopsis* populations differed in SA and camalexin concentrations and whether these compounds were induced in response to a cold treatment. To do this, we used two plants of each of the 34 genotypes, for a total of 68 plants.

We grew them for 30d and then divided them into two groups. One group was assigned to be cold treated and was placed in an equivalent room with the same light levels, but with an average room temperature of 10^{o}C for 48hr. Control plants were handled in an equivalent manner as that the cold treated plants, but were placed in the normal temperature room at 20^{o}C for 48hr. At the 48hr mark, we excised the rosette of each plant at the base and immediately flash froze the material in liquid nitrogen. The harvest of the cold treatment plants was conducted in the 10^{o}C room to ensure that plants did not receive a different temperature at harvest. Plant material was then measured for SA and camalexin concentrations (Table 20) as described below.

To assess whether the study populations differed in resistance to the growth of a bacterial pathogen, we conducted a second experiment in which we challenged plants with *Pseudomonas syringae pv. tomato* DC3000, hereafter "*Pst* DC3000", which is the most widely-used strain for bioassays with *Arabidopsis* (Hirano and Upper 2000). To assess the effects of both "population" and "genotype within population", we included four replicates of each of eight or nine genotypes for each of the four populations for a total of 216 plants (Table 21). On Day 30 of growth, plants were brought to the lab and three largest leaves on each plant were injected with 0.1ml of the inoculation solution of 1x10^{5} *Pst*DC3000 by blunt syringe, using a standard method (Jakob et al. 2002). The bacterial titer of one randomly selected leaf per plant on two plants per genotype was measured on the fourth day after infection. To measure titers, a disk was removed by hole-punch, surface-sterilized in 70% ethanol for 2s, dried with a sterile paper towel, and ground in an Eppendorf tube containing 200µl of 10mM $MgSO_4$ buffer. The homogenate was diluted by 1:1000 and by 1:100000 in buffer. A 50µl aliquot of each dilution was then spread on plates containing King's B medium with 50ug/L rifampycin, incubated for 2d at 28^{o}C, and then counted by eye to determine the number of colonies. This number was multiplied by 20 to determine leaf

bacterial titer (Jakob et al. 2002). Symptoms were scored on the remaining four replicates per genotype as percent of inoculated leaf tissue that was yellow or senesced at 4d post infection.

To determine whether leaf SA and camalexin concentrations could be induced by bacterial infection, we conducted a third experiment in which we grew the full set of genotypes for each of the four populations and treated two replicate plants of each genotype with *Pst* DC3000, with an additional two replicate plants of each genotype as a control, for a total of 136 plants (Table B3). Three largest leaves on each plant were injected with 0.1ml of $1x10^5$ *Pst*DC3000 solution for infection plants, while control plants were inoculated with 0.1ml of the 10mM $MgSO_4$ buffer as a mock control. After 48hr of bacterial treatment, the three inoculated leaves were harvested in liquid nitrogen, with two leaves for analysis of leaf SA and camalexin concentrations as described below and one leaf for further DNA extraction.

3.2.4 Measurement of leaf SA and camalexin concentrations

All leaf samples were stored in a -80^{o}C freezer and then lyophilized to dryness in a freeze-dryer prior to analysis. To extract and analyze leaf concentrations of SA and camalexin, we followed a standard protocol (Dewdney et al. 2000) that we have used successfully in previous work (Mukherjee et al. 2010). Approximately 6-20mg of tissue was weighed, pulverized, and suspended in 3ml of 90% methanol. Samples with less than 5mg of tissue available were excluded. A total of 3 samples in the cold experiment and 14 samples in bacterial infection experiment did not reach the tissue requirement threshold for analysis. We added an internal control of 1µg of O-Anisic Acid (Sigma # 169978) to each sample tube (100µl of a 10µg/ml solution in 100% methanol), vortexed to resuspend the tissue, and rocked the tubes in a shaker at room temperature for 24hr. We then transferred the liquid to a new tube, resuspended the pellet

in 3ml of 100% methanol, vortexed, rocked the tubes again for 24hr, and combined the supernatant fractions.

Because SA exists in plants with two forms, we then split each sample. The first aliquot was used to measure free SA, and the second was used to measure total SA, which includes the large portion of SA that is conjugated to sugar (SA *O-β* –glucoside). Camalexin is present in a single form and thus could be quantified in both sets of samples. We split each sample in equal volumes into two screwcap tubes and placed the tubes in a fume hood until dry (roughly 24hr later). The aliquot for measurement of total SA received 40U of b-glucosidase enzyme (Sigma # 0395) in 400µl of 100mM sodium acetate buffer (pH 5.5) which cleaves the sugar from SA glucoside, thus providing an estimate of total SA present in the sample (free plus glucoside-conjugated). The other aliquot received the 400µl of buffer but no enzyme. All samples were incubated overnight at 37^{o}C and then all received 400µl of 10% trichloroacetic acid. All samples were then partitioned twice with 1ml of an organic extraction solvent (100:99:1 of ethylacetate: cyclopentane: 2-propanol), vortexing each time before collecting the two organic phase fractions in a centrifuge tube. We then placed the tubes in a fume hood until dry (24 to 48hr). We resuspended samples in 600µl of 55% methanol, vortexed, and placed samples in a rocker overnight. We centrifuged samples at 5000g for 15min, transferred the supernatant to 0.2µm nylon spin-prep membrane filters (Fisher #07-200-389), and centrifuged at 14,000g for 5min. Concentrations of SA were then measured by high performance liquid chromatography (HPLC) on an HP1100 system with a 4.6 x 150mm Agilent Eclipse XDB C-18 column and fluorescence detector (Excitation at 301nm and Emission at 412nm for SA, Excitation at 301nm and Emission at 386nm for camalexin, Excitation at 301nm and Emission at 365nm for O-Anisic Acid). Solvent flow was 1ml/min, beginning with 30% of 100% methanol and 70% of 0.5% acetic acid

for five minutes, increasing to 40% methanol at 7.5min and 60% methanol at 15min, returning to 30% methanol at 18min. Concentrations (μg/g leaf dry mass) of free SA, total SA, and camalexin were calculated as the peak area of each compound divided by the product of the peak area of the O-Anisic acid internal standard and sample mass (Fig. 32).

3.2.5 Determination of allelic variation at the ACD6 locus (At4g14400)

DNA was obtained from frozen leaf tissue ground in liquid nitrogen with a pestle in a 1.5ml tube. DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen #69504) according to the manufacturer's instructions. DNA concentration was measured on a Nanodrop 2000 Spectrophotometer. Purified DNA was then amplified using standard PCR methods in a PTC 200 Thermal Cycler. Briefly, each reaction contained 4.5μL 5X buffer (Promega #M792A), 16.0μL ddH_2O, 0.6μL 10mM dNTP, 0.6μL 10μM forward and reverse primers, 2μL of approximately 25ng/μL template DNA, and 0.3μL 5U/μL GoTaq DNA Polymerase (Promega #M830A), for a final volume of 22.0μL. Reaction conditions were as follows: 94°C, 3min; 36X (94°C, 30sec; variable annealing temperature depending on primer used, 30sec; 72°C, 1min); 72°C, 7min. Primers amplifying the ACD6 gene have been described (Todesco et al. 2014). One primer set amplifies the KZ-10-like allele (F: 5'…GCTCATGGCGGTCATACAC…3', R: 5'…TACCGTCTTGGGGAGGAAGT…3'); a 55°C annealing temperature was used in PCR reactions. The second primer set amplifies both Col-0-like alleles as well as Est-1-like alleles (F: 5'…TGGCCACTAACCCAACTCTC…3', R: 5'…GAGCCAGTCTCATCAAAATCG…3'); a 52°C annealing temperature was used. The Est-1-like allele possesses an XmnI restriction site in the middle of the target amplification region. Therefore, to distinguish between the two alleles, PCR products were cut for 1.5hr at 37°C with XmnI restriction enzyme (New England BioLabs

#R0194). PCR products were run on a 1% agarose gel with 0.5μg/mL ethidium bromide and visualized on a Gel Doc EQ system. Finally, to assess nucleotide differences at two critical codons in the coding region of ACD6, we sequenced PCR products from four genotypes from the VIE population (Lines 3, 11, 12, and 20), as well as the Est-1 and Col-0 genotypes, as positive and negative controls, respectively. These sequences were then submitted to the EMBL database (Accession # HG934313-HG934318).

3.2.6 Statistical analysis

Leaf constitutive concentrations (C) of camalexin and SA were analyzed by nested ANOVA in which the predictor variables were population and genotype, with genotypes nested within population. The induction response (R) of plants was calculated as the difference between the induced level (I) and the constitutive level (C), using the equation $R = I - C$. This approach was recommended as the best in a recent review (Morris et al. 2006). T-tests were used then to determine whether R was significantly greater than zero. Statistical analyses were performed using the Minitab 16 software program. Further analysis on correlation between constitutive (C) vs. induction (R) of camalexin was validated with a permutation test in R program (Morris et al. 2006).

3.3 RESULTS

Under common garden conditions in the initial experiment, we found that genotypes from population VIE had hyper-accumulation of camalexin in leaves, producing roughly 20-fold more

camalexin relative to genotypes from the other three populations ($F_{3,32}$=9.6, P<0.001, Fig. 4A) and about 60% more free SA ($F_{3,32}$=3.4, P=0.029, Fig. 4E). The difference in mean total SA among populations was not significant in the first experiment according to the nested ANOVA ($F_{3,32}$=1.0, P=0.38, Fig. 4C, Table 20).

Treatment of plants with 48hr of exposure to a temperature of 10^{o}C had no overall effect on the induced expression of camalexin (T=-0.49, P=0.63), total SA (T=1.02, P=0.32), or free SA (T=1.47, P=0.15). The four populations were also not detectably different in their lack of response in camalexin expression ($F_{3,29}$=1.8, P=0.17, Fig. 4B), total SA ($F_{3,29}$=1.1, P=0.35, Fig. 4D), or free SA ($F_{3,29}$=0.5, P=0.66, Fig. 4F, Table 20).

Plants from VIE showed the most resistance to the growth of the virulent bacterial pathogen, *Pst* DC3000 (Fig. 5, Table 21). The bacterial titers in leaves of plants from the VIE population achieved a 12% lower log titer relative to the other three populations ($F_{3,32}$=6.2, P=0.002, Fig. 5A). Leaf disease symptoms were also less prevalent for genotypes from the VIE population, with plants exhibiting a 14.7% lower amount of disease symptoms in the inoculated leaf at 4 days post infection relative to the other three populations ($F_{3,32}$=9.1, P<0.001, Fig. 5B).

Treatment of plants with 48hr of exposure to bacterial infection by *Pst* DC3000 (Fig. 6, Table 22) led to dramatic overall increases in leaf concentrations of camalexin (T=9.4, P<0.001, Fig. 6B), total SA (T=6.77, P<0.001, Fig. 6D), and free SA (T=4.24, P<0.001, Fig. 6F). The induction responses were roughly 6-fold, 2-fold, and 1.5-fold for leaf camalexin, total SA, and free SA, respectively (Table 22). The mock controls showed once again that genotypes from VIE constitutively produced significantly more camalexin ($F_{3,30}$=33.7, P<0.001, Fig. 6A), relative to genotypes from the other three populations. In this experiment, constitutive expression of total SA was significantly higher in the VIE population than in the BAR and ALE populations

($F_{3,30}$=5.7, P=0.003, Fig. 6C), while free SA did not exhibit a significant effect ($F_{3,30}$=2.2, P=0.10, Fig. 6E). Among the genotypes in the VIE population, VIE-1, VIE-12, and VIE-19 had significantly higher constitutive concentrations of camalexin ($F_{6,35}$=56.7, P<0.001, Fig. 7A), total salicylic acid ($F_{6,13}$=12.8, P=0.002, Fig. 7C), and free salicylic acid ($F_{6,13}$=4.7, P=0.031, Fig. 7E) relative to the other genotypes.

Camalexin induction following bacterial infection was not predicted by constitutive levels of camalexin when genotypic averages of untransformed data were assessed ($F_{1,32}$=1.7, P=0.19, Fig. 8A). Following log transformation, there was a strong negative correlation (r=-0.86) between mean camalexin induction response and constitutive concentration ($F_{1,32}$=83.7, P<0.0001, Fig. 8B), but it is important to note that these two axes are autocorrelated and some degree of negative correlation would be expected even in the absence of an actual relationship (Morris et al. 2006). To account for the autocorrelation, we first regressed a camalexin response value obtained from the second replicate only against the constitutive values measured on the first replicate only, for which we had data for 17 of the genotypes and here a weak, but significant, negative correlation was still observed ($F_{1,16}$=15.5, P=0.001, Fig. 8C). We also used a permutation test approach (Morris et al. 2006) and found that the observed correlation coefficient of -0.86 from the full was significantly more negative than 99.5% of the possible correlation coefficients that could be calculated from any permutation of the dataset P=0.0049, Fig. 34).

Leaf bacterial titers were negatively correlated with log transformed leaf constitutive camalexin concentration (C, $F_{1,31}$=4.8, P=0.037, Fig. 9A) and slightly more strongly with leaf induced camalexin concentration (I, $F_{1,31}$=5.3, P=0.027, Fig. 9B). Induced camalexin

concentration alone explained 15.2% of the variation in leaf bacterial titers and this value was only increased to 20.9% by addition of constitutive camalexin concentration to the model.

To determine whether variation at the ACD6 locus was responsible for the observed hyper-accumulation of SA and camalexin in the VIE population, we first amplified the 3' end of the ACD6 gene including part of the 3' UTR and used a diagnostic test with the XmnI restriction enzyme, which has a binding site in the Est-1 hyper-active allele of this gene, but not in the normal Col-0 allele (Table 20). We found that XmnI cut the fragment from two genotypes from the BAR population (genotypes #3 and #15, Fig. 34), three genotypes from the HOR population (genotypes #1, 2, and 19, Fig. 35), and in three Est-1 positive control plants (Fig. 34), but none from the VIE population (Fig. 34). Three of the four populations were polymorphic at the ACD6 locus (Table 1), indicating a substantial amount of genetic variation in these populations. Because the causal codon differences are some distance from the restriction site, we also sequenced fragments from four VIE genotypes and found that they matched the sequence of the normally functioning Col-0 allele and not the sequence of the hyper-accumulation Est-1 allele (Table 2). Finally, we examined the published genome sequence of the VIE-0 genotype (Cao et al. 2011) and found that it has the normal Col-0 allele at the ACD6 locus. Thus the variation in camalexin expression is not directly related with the Est-1 allele variation at ACD6 locus.

3.4 DISCUSSION

Here, we asked how concentrations of the important plant defense compound, camalexin, vary in four natural populations of Arabidopsis thaliana from the Iberian Peninsula. In a series of

common garden experiments, we found that genotypes from VIE constitutively expressed camalexin, whereas the other three populations did not. These findings for camalexin were robust in that they were measured in two independent large experiments that included at least eight genotypes per population. While other phenolic and indolic compounds have previously been shown to vary geographically across different populations within a species (Alonso-Amelot et al. 2004, Zidorn et al. 2005), leaf camalexin and SA concentrations have not been compared much at the population level [but see (Rowe and Kliebenstein 2008, Chan et al. 2010, Chan et al. 2011)]. Because these compounds have important roles in plant defense against pathogens (Vlot et al. 2009), our finding of population level differences in leaf camalexin and SA concentrations may reflect important differences in the environmental stresses experienced across natural populations.

Plant defense theory predicts that induction responses of plant defenses should be negatively correlated with constitutive concentrations of these same plant defenses (Zangerl and Bazzaz 1992). Given that the constitutive levels of camalexin differed considerably among genotypes and were highest in the VIE population (Fig. 6A), the theory would predict therefore that these genotypes would have a lower induction response. We did find some support for this hypothesis using two different approaches outlined in a recent paper (Morris et al. 2006). First, there was a significant negative correlation when the dataset was split into halves and one set of replicates was used to calculate the induction response and the other set of replicates was used to calculate the constitutive values (Fig. 8C). Second, the observed correlation coefficient of r=-0.86 was significantly more negative than 99.5% of the possible correlation coefficients that could be generated from permutations of the dataset. These results therefore join the

accumulating evidence of such tradeoffs in defense strategies within plant populations (Kempel et al. 2011).

The only described genetic basis for hyper-accumulation of camalexin in plants has been due to variation at the ACD6 locus (Todesco et al. 2010). In that case, a number of wild lines, including the Est-1 genotype, possess a defective allele for the well-known ankyrin repeat protein-encoding gene, Accelerated Cell Death 6 (ACD6). That camalexin hyper-accumulation phenotype in those lines has been conclusively linked to differences at two codons in the Est-1-like allele that trigger plant constitutive hyper-accumulation of SA and camalexin, even in the absence of enemies (Todesco et al. 2010). However, our data suggested that the Est-1 allele could not explain our leaf camalexin phenotypes. First, our restriction analysis showed that none of the genotypes from the VIE population possessed the cutting site for the XmnI restriction enzyme at the ACD6 locus, whereas the hyperactive Est-1 allele does possess the cutting site. Moreover, when we sequenced DNA at the ACD6 locus for four VIE genotypes, we found that none of them possessed the causal nucleotide polymorphisms. Finally, our assessment of the published sequence for the Vie-0 genotype (Cao et al. 2011) revealed that it also lacks the Est-1 allele at this locus. Thus, this difference in defense chemistry among the four Arabidopsis populations possesses an unknown genetic basis.

Plant defense responses are incredibly sophisticated and involve the activity of a large number of proteins, including several in the ankyrin-repeat domain class that includes ACD6 (Becerra et al. 2004) and the better-known NPR1 (Pieterse and Van Loon 2004). Natural defects in these or many other proteins, such as the LRR class of R-genes, could cause genotypes in the VIE population to constitutively express camalexin. We did find five genotypes in the population set that are likely to possess the Est-1 allele at the ACD6 locus and interestingly none of these

exhibited the constitutive accumulation of camalexin. This suggests that the phenotype associated with the hyperactive Est-1 allele can be masked by other variation in these genotypes. Further studies are necessary to understand the dynamics present in these structured natural populations.

While a small number of previous studies have found changes in leaf SA concentration in response to experimental manipulation of temperature (Leyva et al. 1995, Kaplan et al. 2004), we did not observe any effect of our 48hr duration cold treatment of 10oC applied at 30d of plant growth. Whether these plants would respond to a cold treatment that was applied for a longer duration or at a different developmental stage remains unknown. The VIE population does experience the coldest temperatures among the four populations studied (Table 1), but a number of other environmental factors are likely to differ among these four sites as well, including differences in ambient levels of UV radiation. For these reasons it is not possible currently to link the population level differences that we found to any particular environmental factor. It is clear however that all four populations and 34 genotypes included in the study are capable of producing camalexin. We can conclude this because all of the genotypes responded to 48hr of infection by the virulent bacterial pathogen, Pseudomonas syringae pv. tomato DC3000 with robust and dramatic increases in leaf camalexin concentration within the 48hr period (Fig. 6B).

Our four study populations are part of a larger published set of 17 populations collected along an elevation gradient from the Mediterranean shore into the Pyrenee Mountains (Montesinos-Navarro et al. 2011). In that previous study, the number of leaves at bolting was nearly identical for BAR, HOR and ALE, while VIE exhibited nearly 50% higher leaf numbers at bolting compared to the other three populations. However, the rate of leaf initiation varied clinally with the combination of life history stage and environmental conditions in that study.

VIE initiated the most leaves pre- and post-vernalization, while the low altitude populations initiated substantially more leaves during the 5°C vernalization period imposed in that study (Montesinos-Navarro et al. 2011). Thus, VIE differs in leaf initiation rates compared to the other study populations, but in a complex way that cannot be readily compared to the leaf initiation results reported elsewhere (Todesco et al. 2010). It appears that the elevated constitutive expression of free SA and camalexin observed in this study has a unique mechanism of genetic control compared to the genotypes assayed by Todesco et al. 2010, and may not have the same trade-offs in leaf initiation rate. The genetic basis of the observed differences between VIE and our other three populations deserves further study.

Our data suggests the possibility that natural populations of Arabidopsis thaliana vary in their constitutive expression of secondary compounds in general and possess the ability to elevate the expression of these secondary compounds to infection by the bacterium, Pseudomonas syringae pv tomato DC3000. The climate conditions of the four populations vary in their temperature, precipitation and UV radiation. These factors together might differentially activate the defense pathway. Assay of SA and camalexin content of additional populations along an elevational gradient will be necessary to test this hypothesis. Should a general association between high elevation sites and high constitutive phytoalexin production be observed, such elevational gradient patterns may provide insight into the consequences of climate change for plant populations. Such data may be useful in understanding how plant resistance will be affected by warming climate (Boland et al. 2004). In that case, genotypes with highest constitutive expression of these compounds, such as genotypes from VIE, could be used as natural samples with higher plant resistance for study of the correlation between particular climate variables and plant defense ability.

On the other hand, these secondary compounds, especially SA, also have significant roles on plant growth, development and other stresses tolerance (Vlot et al. 2009). The endogenous level of SA could vary depending developmental stages and environmental conditions. For this reason, comparisons should be made cautiously across plant life cycle and experimental manipulations. Moreover, constitutively expressed SA could also trade-off with plants' ability to withstand abiotic stresses like freezing or heat tolerance (Taşgín et al. 2003, López-Delgado et al. 2004, Mateo et al. 2006). The adaptive benefits of constitutive and induced expression of SA and camalexin remain to be discovered in these natural populations on the Iberian Peninsula. The goal of future work will be therefore to establish how genetic variation structures the phenotypic variation in this unique set of natural populations.

Table 1. Elevation, coordinates, annual mean temperature, and allelic assessment at the ACD6 locus of four source populations of *Arabidopsis thaliana* from the Iberian Peninsula

Elevation(m)	Population	Long	Lat	Annual Mean Temp (°C)	Allelic variation at the ACD6 Locus		
					Est-1-like (%)	KZ-10-like (%)	Col-0-like (%)
429	BAR	2.1278	41.4322	15.4	33.3%	66.7%	0.0%
431	HOR	2.6202	41.6645	13.9	37.5%	62.5%	0.0%
1225	ALE	1.3187	42.4105	8.5	0.0%	100.0%	0.0%
1600	VIE	0.7606	42.6256	5.1	0.0%	16.7%	83.3%

Table 2. Partial sequence from the coding region at the ACD6 locus (Chr. 4, bases 8,297,993 to 8,298,425) for four maternal lines from the VIE population along with sequence obtained from the Est-1 and the Col-0 accessions, as controls. The four VIE genotypes share the sequence of the Col-0 genotype at the two critical codon positions in ACD6 (underlined and highlighted), rather than that of the hyperactive Est-1 genotype (Tsuji et al. 1992). These sequences are available in the EMBL database (Accession # HG934313-HG934318)

>ACD6 VIE-3
GTTCTGTTGCAACAATATGTACTCTTATTTGGGCGCAGTTGGGTGATCTA**GCA**CTCATCCTCAAATCCTTACATGTGGCCTTGCCC
TTACTACTTTTTTCATTACTATGCATGCCCGTGGCATTCCTTTTTGGCGTGATCACTGCAATTGCCCATGTGAAATGGCTTTTAGT
CACCATTAGCATTATATCTGGTGGATTCTTCCTTTTCGCAATCTTTATCCTTGGCCCTCACGTCATGCTACAGCGCTCACAC**CTT**C
CGCCCAGTTCTGGTATATTTCTCAAGACTTTTATGCTGACTATAGACATATCTGAGTTGTTTGTGATTTTGATCAAAGCTTGTTTT
GGTTGTGTGGCGTGTTCCGAATAAATCATCAAAAGTTTATAGATCAAAGGATATTTTTCTTCTCTTTTTTTTTTCTTTCAAAAAGC
TAC

>ACD6 VIE-11
GTTCTGTTGCAACAATATGTACTCTTATTTGGGCGCAGTTGGGTGATCTA**GCA**CTCATCCTCAAATCCTTACATGTGGCCTTGCCC
TTACTACTTTTTTCATTACTATGCATGCCCGTGGCATTCCTTTTTGGCGTGATCACTGCAATTGCCCATGTGAAATGGCTTTTAGT
CACCATTAGCATTATATCTGGTGGATTCTTCCTTTTCGCAATCTTTATCCTTGGCCCTCACGTCATGCTACAGCGCTCACAC**CTT**C
CGCCCAGTTCTGGTATATTTCTCAAGACTTTTATGCTGACTATAGACATATCTGAGTTGTTTGTGATTTTGATCAAAGCTTGTTTT
GGTTGTGTGGCGTGTTCCGAATAAATCATCAAAAGTTTATAGATCAAAGGATATTTTTCTTCTCTTTTTTTTTTCTTTCAAAAAGC
TAC

>ACD6 VIE-12
GTTCTGTTGCAACAATATGTACTCTTATTTGGGCGCAGTTGGGTGATCTA**GCA**CTCATCCTCAAATCCTTACATGTGGCCTTGCCC
TTACTACTTTTTTCATTACTATGCATGCCCGTGGCATTCCTTTTTGGCGTGATCACTGCAATTGCCCATGTGAAATGGCTTTTAGT
CACCATTAGCATTATATCTGGTGGATTCTTCCTTTTCGCAATCTTTATCCTTGGCCCTCACGTCATGCTACAGCGCTCACAC**CTT**C
CGCCCAGTTCTGGTATATTTCTCAAGACTTTTATGCTGACTATAGACATATCTGAGTTGTTTGTGATTttGATCAAAGCTTGTTTT
GGTTGTGTGGCGTGTTCCGAATAAATCATCAAAAGTTTATAGATCAAAGGATATTTTTCTTCTCTTTTTTTTTTCTTTCAAAAAGC
TAC

>ACD6 VIE-20
GTTCTGTTGCAACAATATGTACTCTTATTTGGGCGCAGTTGGGTGATCTA**GCA**CTCATCCTCAAATCCTTACATGTGGCCTTGCCC
TTACTACTTTTTTCATTACTATGCATGCCCGTGGCATTCCTTTTTGGCGTGATCACTGCAATTGCCCATGTGAAATGGCTTTTAGT
CACCATTAGCATTATATCTGGTGGATTCTTCCTTTTCGCAATCTTTATCCTTGGCCCTCACGTCATGCTACAGCGCTCACAC**CTT**C
CGCCCAGTTCTGGTATATTTCTCAAGACTTTTATGCTGACTATAGACATATCTGAGTTGTTTGTGATTTTGATCAAAGCTTGTTTT
GGTTGTGTGGCGTGTTCCGAATAAATCATCAAAAGTTTATAGATCAAAGGATATTTTTCTTCTCTTTTTTTTTTCTTTCAAAAAGC
TAC

>ACD6 Est-1
GTTCTGTTGCAACAATATGTACTCTTATTTGGGCGCAGTTAGGTGATCCA**AAC**CTCATCCGCAAATCCTTACATGTGGCCTTGCCC
TTACTACTTTTTTCATTACTATGCATGCCCGTGGCATTCCTTTTTGGCGTGATCACTGCAATTGCCCATGTGAAATGGCTTTTAGT
CACCATTAGCATTATATCTGGTGGATTCTTCCTTTGCGCAATCTTTATCCTTGGCCCTCACGTCATGCTACAGCGGTCATAC**TTT**C
CGCCCAGTGCTGGTATATATCTCAGGACTTTTATGCTGACTATAGACATATCTGAGTTTTTTGTGCGTAAGATCAAAACTTGTTTT
GGTTGTGTGGCGTGTGAATAAATCATCAAAAGTTTATAGTTCAAAGGATATGTTTCTTCTCTTTTTTTTTTCTTTCAAAAAGCTAC

>ACD6 Col-
0GTTCTGTTGCAACAATATGTACTCTTATTTGGGCGCAGTTGGGTGATCTA**GCA**CTCATCCTCAAATCCTTACATGTGGCCTTGCC
CTTACTACTTTTTTCATTACTATGCATGCCCGTAGCATTCCTTTTTGGCGTGATCACTGCAATTGCCCATGTGAAATGGCTTTTAG
TCACCATTAGCATTATATCTGGTGGATTCTTCCTTTTCGCAATCTTTATCCTTGGCCCTCACGTCATGCTACAGCGCTCACAC**CTT**
CCGCCCAGTTCTGGTATATTTCTCAAGACTTTTATGCTGACTATAGACATATCTGAGTTGTTTGTGATTTTGATCAAAGCTTGTTT
TGGTTGTGTGGCGTGTTCCGAATAAATCATCAAAAGTTTATAGATCAAAGGATATTTTTCTTCTCTTTTTTTTTTCTTTCAAAAAG
CTAC

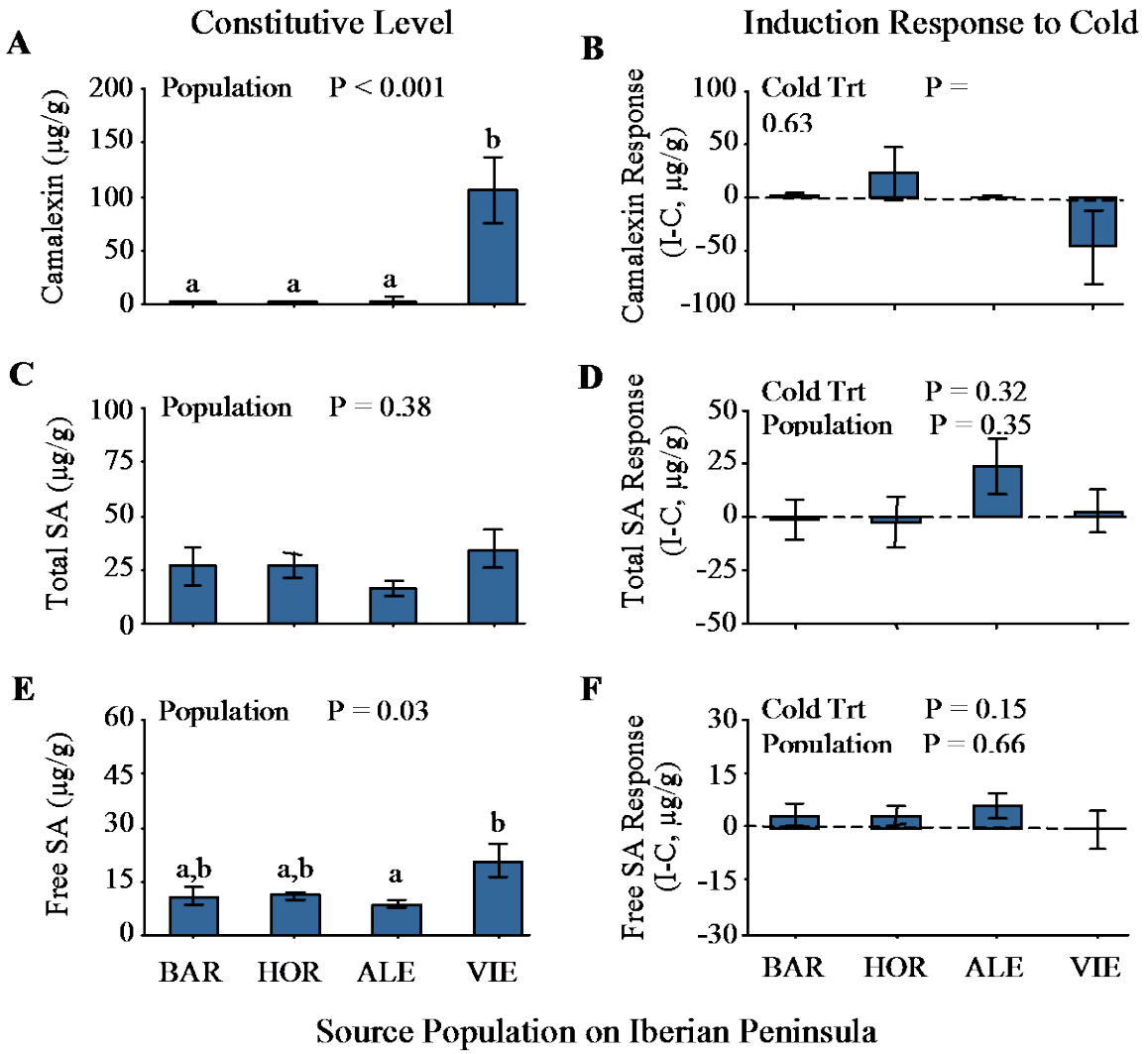


Figure 4. Population mean leaf concentrations (µg/g dry weight) of A) constitutive camalexin, B) induction response of camalexin, C) constitutive total salicylic acid, D) induction response of total salicylic acid, E) constitutive free salicylic acid, and F) induction response of free salicylic acid of *A. thaliana* from eight or nine genotypes from four source on the Iberian peninsula. Shown are means (+/-1SE). Constitutive levels (C) were measured on plants grown at the standard temperature of 20°C. Plants in the 10°C cold treatment (I) were separated from the control plants on Day 30 and placed at the lower temperature for 48hr prior to collection of leaves from all plants.

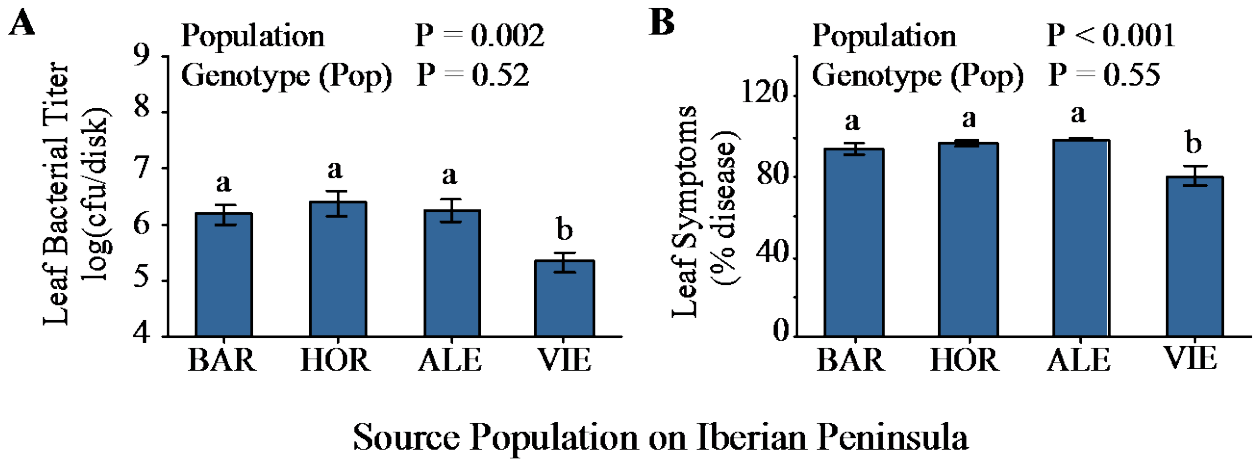


Figure 5. Population differences in A) leaf titers of Pseudomonas syringae pv. tomato DC3000 (Pst DC3000) and B) leaf disease symptoms from a common garden challenge of four populations of A. thaliana on the Iberian peninsula. Bacterial titers were determined at four days post infection from leaves inoculated with 1 x 10^5 colony forming units (cfu) of Pst DC3000 using standard methods (Traw et al. 2007). Symptoms were scored as percent of inoculated leaf tissue that was yellow at 4 days post infection. Shown are means (+/-1SE) for three week old plants representing at least eight genotypes per population. Letters indicate a significant difference among populations at P=0.01.

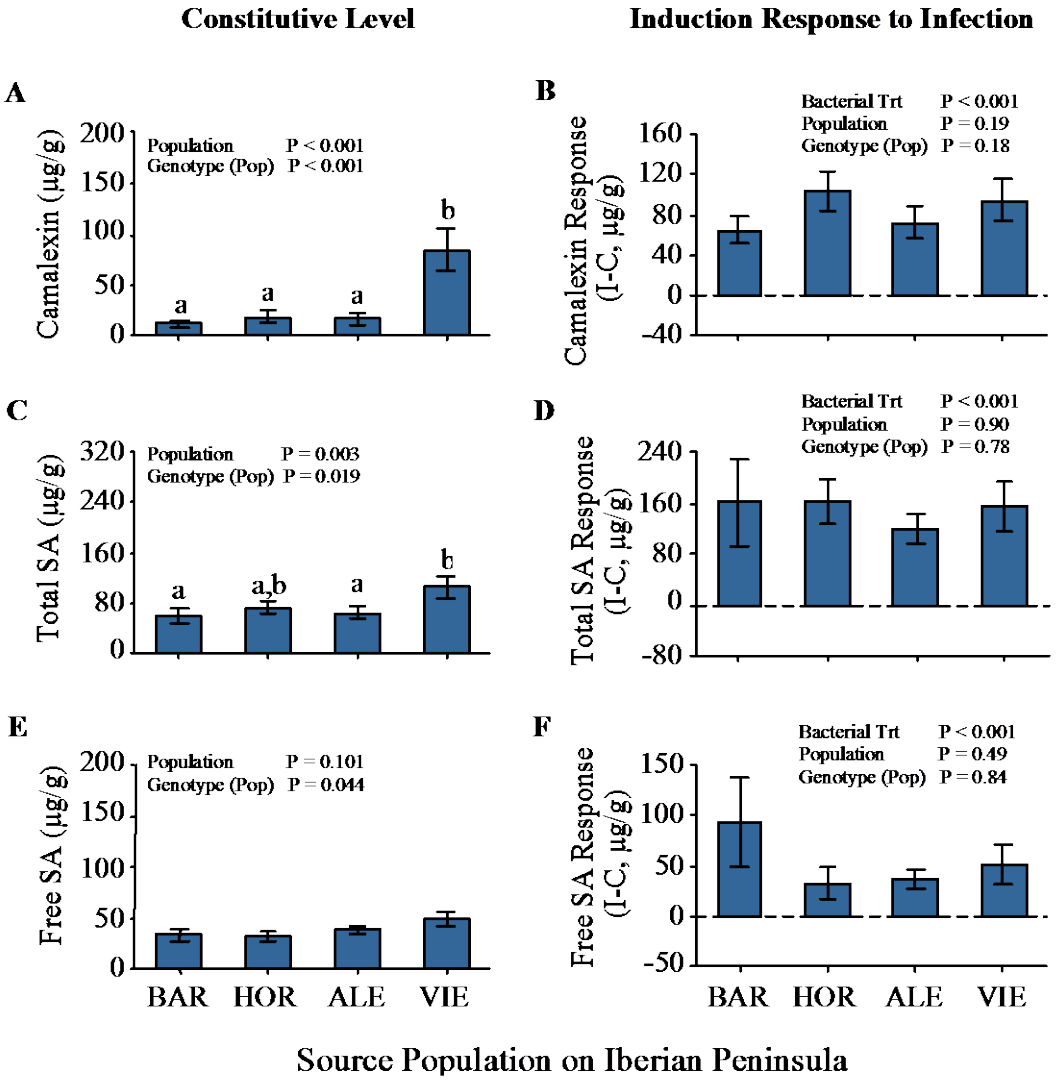


Figure 6. Population mean leaf concentrations (µg/g dry weight) of A) constitutive camalexin, B) induction response of camalexin, C) constitutive total salicylic acid, D) induction response of total salicylic acid, E) constitutive free salicylic acid, and F) induction response of free salicylic acid of *A. thaliana* on the Iberian peninsula. Each mean (+/-1SE) represents the average of two replicates for each of eight or nine genotypes per population measured on plants infected with a $1x10^5$ cfu solution of *Pseudomonas syringae* or mock inoculation solution containing the 10mM $MgSO_4$ buffer only on Day 30 of plant growth. Leaves were harvested for chemical analysis at 48hr post infection. Letters indicate a significant difference among populations at P=0.05.

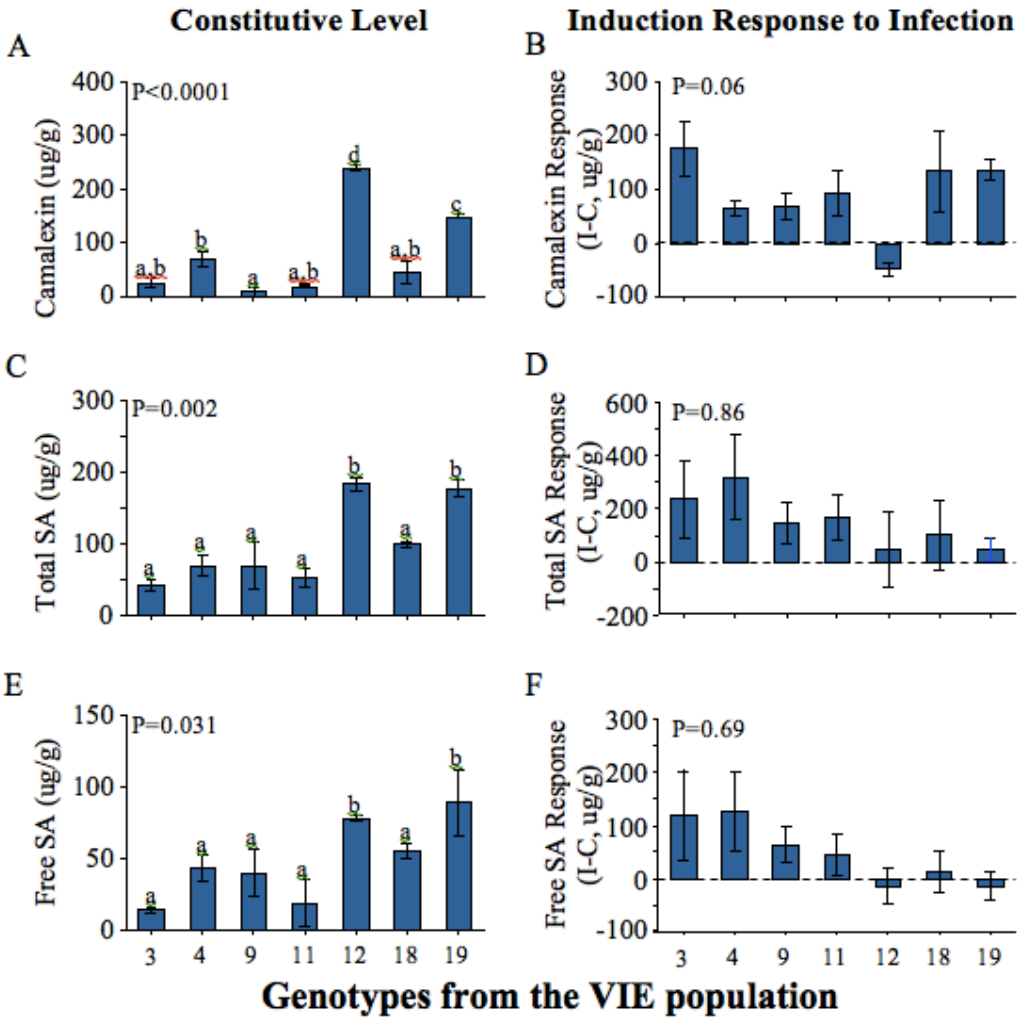
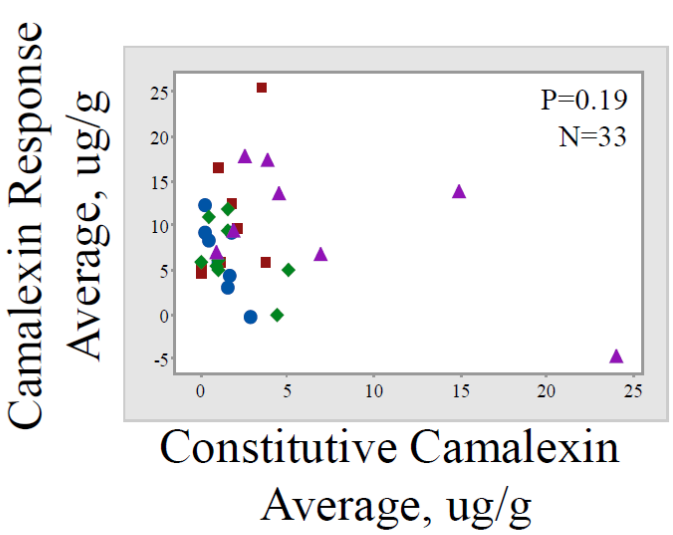
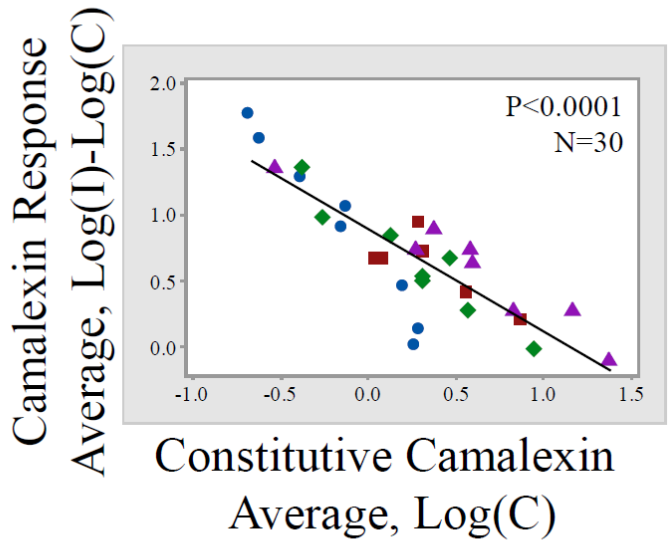


Figure 7. Genotype mean leaf concentrations (µg/g dry weight) of A) constitutive camalexin, B) induction response of camalexin, C) constitutive total salicylic acid, D) induction response of total salicylic acid, E) constitutive free salicylic acid, and F) induction response of free salicylic acid of *A. thaliana* on the Iberian peninsula. Each mean (+/-1SE) represents the average of two replicates for each of eight or nine genotypes per population measured on plants infected with a $1x10^5$ cfu solution of *Pseudomonas syringae* or mock inoculation solution containing the 10mM $MgSO_4$ buffer only on Day 30 of plant growth. Leaves were harvested for chemical analysis at 48hr post infection. Letters indicate a significant difference among populations at P=0.05.

A


Camalexin Response
Average, ug/g
P=0.19
N=33
25
20
15
10
5
0
-5
0
5
10
15
20
25
Constitutive Camalexin
Average, ug/g

B


Camalexin Response
Average, Log(I)-Log(C)
P<0.0001
N=30
2.0
1.5
1.0
0.5
0.0
-1.0
-0.5
0.0
0.5
1.0
1.5
Constitutive Camalexin
Average, Log(C)

C

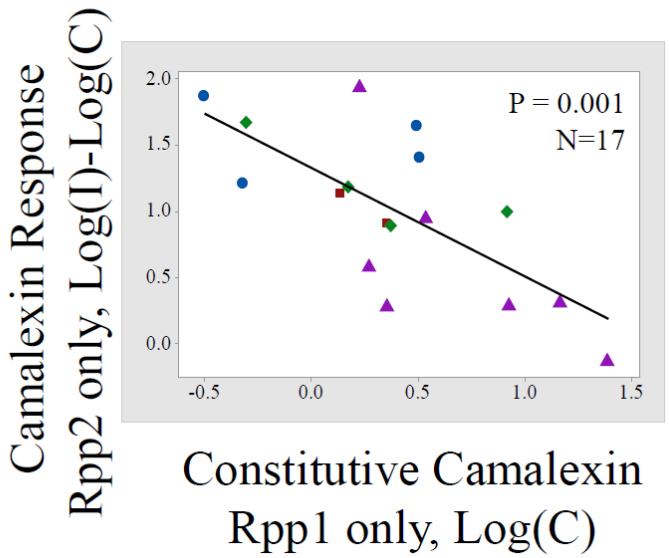

Camalexin Response
Rpp2 only, Log(I)-Log(C)
P = 0.001
N=17
2.0
1.5
1.0
0.5
0.0
-0.5
0.0
0.5
1.0
1.5
Constitutive Camalexin
Rpp1 only, Log(C)

Figure 8. Scatterplots of the induction response of leaf camalexin as a function of constitutive camalexin concentrations among all 34 genotypes. Source populations are indicated as follows: ALE (diamond), BAR (circle), HOR (square), and VIE (triangle). Genotypic means are shown for A) untransformed data and B) log transformed data and were calculated from two replicate plants at two days post inoculate with either a 1x105 cfu solution of Pst DC3000 in 10mM MgSO4 buffer or a mock control containing only the 10mM MgSO4 buffer. Because the y-axis is a function of the x-axis, the errors associated with these axes are not independent and a negative correlation is expected even in the absence of a tradeoff [4]. The final plot shows the response calculated only from replicate 2 and the constitutive concentration from replicate 1 for the 17 genotypes where this could be calculated. P-values are shown from least squares regression.

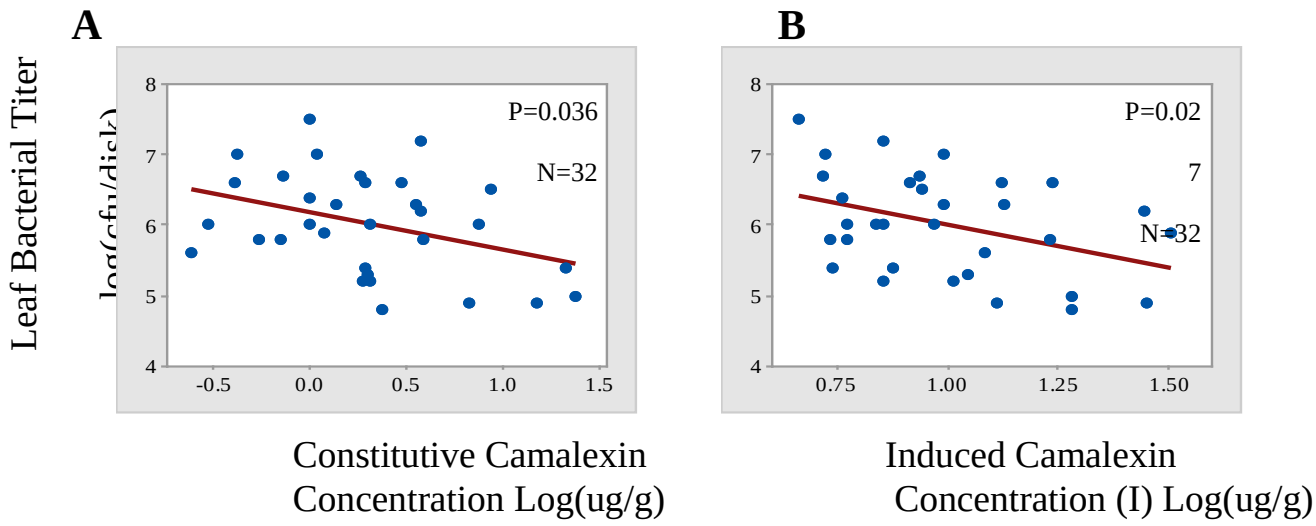


Figure 9. Scatterplot of leaf bacterial titer (log(cfu/disk)) as a function of leaf A) constitutive camalexin concentration(C) log(ug/g dry weight) and B) induced camalexin concentration (I) log(ug/g dry weight). Each circle represents the mean of two replicate plants per genotype. Leaf bacterial titers were determined at 4 days post infection with a 1x105 cfu solution of Pst DC3000. Leaf camalexin concentrations were determined in a separate experiment using tissue collected from plants at 2 days post infection with a 1x105 cfu solution of Pst DC3000 in 10mM MgSO4 buffer (induced) and a mock inoculation solution containing the 10mM MgSO4 buffer (constitutive), respectively. P-value shown from least squares regression.

4.0 NATURAL POPULATIONS OF ARABIDOPSIS THALIANA DIFFER IN SEEDLING RESPONSES TO HIGH TEMPERATAURE STRESS

4.1 INTRODUCTION

Increasing temperatures in many regions of the world may be a defining environmental change in the 21st century. Increasing heat stress is highly likely to lead to shifts in geographic distributions or even to complete extinction of many species (Field *et al.* 2014). Even without current human-driven rapid changes in thermal environments, populations at the warmer edge of their species' geographic range often face strong stresses from high temperature events (Gaston 2009). Variation in thermal environment among sites within species' ranges may result in adaptive variation in thermotolerance. However little is known about the within species ability to evolve thermotolerance responses (Barua *et al.* 2008; Tonsor *et al.* 2008; Paul *et al.* 2011). In this study we examine variation among natural populations of *Arabidopsis thaliana* in their ability to survive and recover from heat stress. We also test the extent to which survival and recovery from heat stress is correlated with the expression of heat shock protein.

Exposure to high temperature can cause severe, irreversible cellular damage and loss of cellular function (Shabala 2012). The threshold temperature at which irreversible damage begins can vary among populations from contrasting in climates (Barua *et al.* 2008). The threshold temperature for damage can also be plastic in response to conditions experienced prior to high

temperature exposure (Wehner *et al.* 1985). When temperatures increase gradually or when plants experience a prior exposure to moderately high temperatures, changes in gene expression ensue (Hannah *et al.* 2006; Larkindale *et al.* 2005; Larkindale and Vierling 2008), leading to greater thermotolerance. This reprogramming and subsequent increase in thermotolerance is termed acclimation or acquired thermotolerance (hereafter AT).

AT has been shown to depend, in part, on the rapid expression of heat shock proteins (Hsps) (Lindquist 1986; Wang *et al.* 2004). Heat causes protein denaturation, disrupting normal protein function. The Hsps act as molecular chaperons to prevent protein aggregation, repair protein damage, and maintain cellular homeostasis. Hsps stabilize protein form and prevent aggregation. The Hsps together recover or protect normal cellular function provided the heat stress is not too extreme (Hong and Vierling 2001). While some other Hsps have been shown to have important roles in the heat shock response and in thermotolerance, to date only Hsp101 has been shown to be essential for AT in plants. Hsp101 has a specific role as a protein machine that disaggregates misfolded proteins (Doyle and Wickner 2009).

This has been demonstrated in both *Arabidopsis thaliana* (Hong and Vierling 2001) and maize (Nieto-Sotelo *et al.* 2002). Hsp101, the cytosol-expressed homologue of the Hsp100/ClpB gene family in *Arabidopsis thaliana*, re-solubilizes and refolds denatured protein. Hsp101 is present in a single copy, Athsp101, in the *Arabidopsis thaliana* genome. To date, nearly all plant studies of Hsp100/ClpB gene products have focused on understanding Hsp101 genetics and biochemistry using genetic derivatives of the most commonly studied genotype of *Arabidopsis thaliana,* Columbia (Col-0), in laboratory settings (Hong *et al.* 2003; Larkindale and Knight 2002; Larkindale *et al.* 2005; Tonsor *et al.* 2008).

Only a little is known about the extent to which natural populations evolve different patterns of Hsp101 expression in response to the differing patterns of heat stress they have experienced in nature. Natural populations of wild species can be important tools for studying the evolution of thermotolerance because they can carry adaptive signatures of selection imposed by their climates of origin. We know of only four studies that directly investigate the relationship between Hsps and thermotolerance in natural plant populations. Barua *et al.* (2003) demonstrated variation among ecotypes of *Chenopodium album* in thermotolerance of photosynthetic electron transport. This thermotolerance variation was strongly associated with variation in chloroplast small Hsp expression and with the thermal environments from which the genotypes were collected (Barua *et al.* 2003). Barua *et al.* (2008) further characterized geographically based variation among genotypes of *Chenopodium album* in Hsp60, Hsp70 and small Hsp expression, finding that expression was greatest in genotypes from habitats that were subject to the greatest fluctuations in temperature. Habitats with high mean and maximum temperatures did not show greater Hsp expression (Barua *et al.* 2008). Similarly, Tonsor *et al.* (2008) tested standard accessions of *Arabidopsis thaliana* whose sites of origin came from a broad latitudinal range. These accessions showed co-variation between latitude of origin and leaf Hsp101 content, with genotypes from warmer latitudes exhibiting lower content (Tonsor *et al.* 2008). Amano *et al.* (2012) compared two species of *Potamogeton (*Potamogetonaceae); one species was heat tolerant, the other was heat-intolerant. *P. malainus* from shallow waters with high temperature fluctuations showed higher thermotolerance while *P. perfoliatus,* from deeper waters with cooler, more constant temperatures had lower basal thermotolerance and could not develop AT because of changes in some of the heat shock element binding sites for HSFA2 (Amano *et al.* 2012). These studies taken together suggest that environments that differ in temperature regimes may

lead in general to evolved differences in heat shock responses. The studies further suggest that evolved responses are likely the result of complex interactions of plants with their local climates. Predictions as to the expected pattern of response are therefore not likely to be accurate. Instead, we chose to simply ask: what are the extant patterns of responses?

Population level studies exploring the relationship between the climate of origin, differentiation in thermotolerance, and the role of changes in gene expression in acquired thermotolerance are needed to improve understanding of optimal heat stress responses in plants. Studies at the population level can provide insights on how populations, from common genetic background, adapted in the past to the novel climates in which they found themselves. Such studies can also help us predict more finely on how plants will respond to future climate shifts. Evolution of heat shock proteins and heat shock response at large geographic scale has been largely conducted in animal species (Feder ME, 1999). In fruit flies, *Drosophila virills* from low latitude showed higher thermotolerance and hsp70 accumulation after 40-41^{o}C heat shock (Garbuz *et al.* 2003), In a marine system, differential response to heat stress in invasive vs. native blue mussels showed the importance of heat shock response to invasive success (Lockwood et al. 2010). Heat shock proteins were found to have different roles in heat stress response, but to have generally been up-regulated (Sørensen *et al.* 2001; Sørensen *et al.* 2005; but see Jensen *et al.* 2009). Here we identify adaptively differing patterns of Hsp expression that correspond with adaptation to contrasting thermal climates in the genomically highly enabled study system, *Arabidopsis thaliana*. These results provide fundamental insights needed for future studies of the genetics, biochemistry, and evolutionary ecology of variation in Hsp expression and thermotolerance in natural populations.

Many climate variables are correlated with elevation (Körner 2007) and elevation gradients create climate gradients across short distances that substantially mimic the broader gradients incorporated in many species' full geographic ranges. Thus studying plants along an elevation gradient can simplify the logistics of climate-plant adaptation studies (Montesinos-Navarro *et al.* 2011). With F. Xavier Picó (La Doñana Biological Station, Seville, Spain) we collected 16 natural populations of *Arabidopsis thaliana* along a climate and elevation gradient in southern Europe extending from near sea level at the Mediterranean coast to near treeline (c. 2200 m above sea level (asl)) in the Pyrenee Mountains. Low elevation sites are hotter and dryer overall, while high elevation sites are cooler and wetter (Montesinos-Navarro *et al.* 2009; Montesinos-Navarro *et al.* 2011; Montesinos-Navarro *et al.* 2012; Wolfe and Tonsor 2014). Because the collection locations were precisely geo-referenced, we obtained accurate local climate data from geo-referenced climate databases. In addition, these populations appear to share common ancestry. Their co-ancestry results from dispersal across the landscape of northern Spain after emergence from a glacial refugium (Picó *et al.* 2008). These natural populations therefore allow the exploration of a series of important ecological and evolutionary questions regarding adaptive divergence from common ancestry. These local populations have evolved functional differences that appear to adapt them to local climates. Traits associated with life history, such as biomass allocation, fecundity, developmental time, and demographic traits, such as seed dormancy and timing of seed germination, showed clinal variation along the elevation gradient (Montesinos-Navarro *et al.* 2009; Montesinos-Navarro *et al.* 2011; Montesinos-Navarro *et al.* 2012). Low elevation populations evolved an early flowering strategy that adapts them to spring heat and drought (Wolfe and Tonsor 2014). These populations also showed variation in genetic diversity, with high elevation populations being more genetically diverse than low

elevation populations (Gomaa *et al.* 2011)..In this study we address local population evolutionary adaptive divergence in heat-shock response and heat-shock proteins across a climate gradient. Our study examines mechanisms that may underpin adaptive variation and contributes to growing knowledge on abiotic stress responses in natural plant populations.

In this study, we characterize genetically-based differences among 16 wild-collected populations in their responses to heat challenges, focusing on evolutionary divergence in thermotolerance (survival and post-stress growth) and Hsp101 expression.

We addressed the following hypotheses:

- Hypothesis 1: Populations of *A. thaliana* vary in their responses to high temperature when tested in experimentally controlled heat treatments;
- Hypothesis 2: Hsp101 expression variation is positively related with observed variation in thermotolerance;
- Hypothesis 3: The observed variation in thermotolerance and Hsp101 expression is associated with the climate of population origin and is therefore adaptive.

4.2 METHODS

4.2.1 Source populations

Seeds of *A. thaliana* were field-collected and subjected to at least two rounds of single-seed descent in controlled environment chambers. 64 genotypes, four from each of 16 population locations (Table 3) [also see Fig.3], were chosen for study. The original seed were collected in collaboration with F. Xavier Picó (Montesinos-Navarro *et al.* 2009; Montesinos-Navarro *et al.* 2011). Dr. Picó states that no specific permissions were necessary for any of the 16 collection sites (F.X.P. pers. comm. to S.J.T. 6/30/2014). The population locations are listed in Table 3. All

genotypes have been donated to the *Arabidopsis* Biological Resource Center at Ohio State University (stock CS78884).

For all collections, we quantified Hsp101 expression directly after heat stress (detailed below). In contrast we quantified thermotolerance one week after heat stress. To obtain these disparate data, we planted two full sets of seeds/populations for each of the four treatments, one destined for Hsp101 quantification immediately following treatment, and one that was grown for one week following heat treatment and assayed for seedling survival and root growth.

4.2.2 Planting designs

Our hypotheses are focused mainly on understanding population-level differences. We maximized the accuracy of our population mean estimates within the constraints of total size of the experiment. Estimates of individual line performance were less important, especially given the relatively low within-population genetic variance in this highly selfing species.

Thermotolerance assay planting design. Four complete replicate sets of agar plates were prepared. Each set included two seedlings of each of the 64 Spanish lines, 128 seedlings total. This gave us eight replicate measures of each population's characteristics. Six seeds were planted per plate; the 128 seeds of a set were distributed across 22 plates. Seeds were randomly assigned to plates, but no plate contained two seeds from any one genotype or population. Because the design does not completely fill 6 x 22 = 132 locations, we were able to also include seeds for a pilot study that are not included in this study in some randomly chosen locations. These pilot study seeds will not be mentioned further.

Hsp101 assay planting design. Population-level estimates of Hsp101 expression were based on a single randomly chosen genotype from each population. Because *A. thaliana* is

highly selfing (Abbott and Gomes 1989; Nordborg *et al.* 2002) and highly selfing populations tend toward low amounts of internal genetic variation (Duminil et al. 2007; Loveless and Hamrick 1984; Hamrick and Godt 1996) but see (Platt *et al.* 2010). Each genotype was represented by two replicate plantings, each containing 15-20 seeds; four genotypes were randomly assigned to a plate. This number of seedlings assured sufficient tissue for Hsp101 extraction and quantification.

4.2.3 Heat stress treatments

The most informative heat stress temperature was unknown at the start of the experiment. Virtually all prior heat stress experiments with *A. thaliana* have been conducted on mid-northern European genetic lines. However, Tonsor *et al.* demonstrated a cline in Hsp101 expression with latitude suggesting that populations will differ in the temperatures that represent a heat stress (Tonsor *et al.* 2008). Our study populations are from southern European latitudes (Montesinos-Navarro *et al.* 2009). Temperatures in their sites of origin range from cooler to warmer, unlike temperatures in sites of the standard laboratory lines. Given the unknown nature of temperature responses among our lines we therefore imposed two heat challenges, 42°C and 45°C. Our approach was to heat stress 10-day old seedlings grown on agar plates, and then measure survival, post-stress root growth, and quantify Hsp101 expression.

Arabidopsis seedlings are known to acquire greater heat tolerance with acclimation (AT). The standard acclimation treatment in prior published studies was 3 hrs at 38°C (Hong *et al.* 2003). We therefore compared responses at both 42°C and 45°C for two sets of replicates: those with 38°C acclimation treatment (AT) and thermally naïve control treatment (CT) seedlings maintained at 22°C prior to heat stress. Thus we employed four thermal treatments. In each of

these seedlings were grown at 22°C prior to and following any heat treatments. Our four thermal treatments were: 1) CT42: 42°C for 3hrs; 2) CT45: 45°C for 3hrs; 3) AT42: 3hrs at 38°C, recovery at 22°C for 3hrs, then 3hrs at 42°C; 4) AT45: 3hrs at 38°C, recovery at 22°C for 3hrs, then 3hrs at 45°C.

4.2.4 Growth protocols and heat stress assays

Seeds were surface-sterilized by exposure to chlorine gas for three hours. Seeds were grown on gridded square agar plates containing Mirashige and Skoog nutrient solution. For the *thermotolerance assay*, seeds were placed on the top grid line and plates were vertically oriented to allow measurement of root growth. For the *Hsp101 assay* the plates were divided into four quadrats and seeds of a randomly-chosen genotype were placed in the middle of one of the four quadrats. Plates were oriented horizontally. For all plates in both planting designs, after five days of stratification at 4°C, plates were placed under fluorescent lights and maintained at approximately 22°C with 16 hrs light, 8 hrs dark, except during any of the experimental heat treatments. After seed placement all plates were sealed for the duration of the experiment. Fifteen days after planting, ten days after emerging from stratification, each replicate heat stress set was exposed to one of the four heat stress treatments as mentioned above. The plates were vertically positioned on racks in a large forced-air drying oven to guarantee rapid air movement and conductive heat transfer. Although we did not measure the temperature of individual plates, heat transfer in the oven is very fast and even. In combination with the low mass of the agar plates we are certain that plate and agar temperature equilibrated with oven air temperature within a few minutes of placement in the oven. Those sets intended for the thermotolerance assay were returned to 22°C with 16 hrs light, 8 hrs dark and grown for one week after heat

stress. The sets of plates destined for Hsp101 quantification were harvested immediately after heat stress.

4.2.5 Thermotolerance measures

We measured seedling survival and post-stress root growth, two widely used measures of heat stress effects (Yeh *et al.* 2012). A seedling was counted as dead if all the leaves turned from green to white. Percent seedling survival was calculated as the percentage of living seedlings one week post-stress for each population. Plates were photographed just prior to heat stress and one week post treatment. Root length was traced using NeuronJ (a macro in NIH ImageJ) for roots in the pre- and post-treatment photos. For each seedling, the post-stress root growth was quantified as the length difference between the pre- and post-treatment images.

4.2.6 Hsp101 quantification

All seedlings used in the Hsp101 assay were collected immediately after heat treatment and were used for western blot Hsp101 quantification. Seedlings were collected into microcentrifuge tubes and immediately put into liquid nitrogen. A bulk Hsp101 expression standard was prepared by combining multiple leaves from a variety of genotypes that had been subjected to a 42°C heat treatment. This bulk Hsp101 expression standard was used for comparison across individual gels to minimize gel-to-gel variation. Hsp101 accumulation level was quantified through image analysis of western blots following the procedure of Tonsor et al. (2008). We used N-terminal Hsp101 primary antibody from rabbit (Agrisera, AS07253) as the primary antibody and anti-rabbit antibody as the secondary antibody to capture Hsp101.

4.2.7 Climate quantification

Wolfe & Tonsor combined temperature and precipitation data at the 16 collection sites from the BIOCLIM dataset ((Hijmans et al. 2005 data available at http://www.worldclim.org) and used principal component analysis to reduce the dimensionality of this data set (Wolfe and Tonsor 2014). Two principal components (PCs) were significant based on permutation tests. Their ClimatePC1 explained 75% of the multivariate variance across the 19 BIOCLIM variables while ClimatePC2 explained 17% (See Fig.S3 in Wolfe and Tonsor 2014). ClimatePC1 was most strongly associated with temperature and precipitation (cool and moist vs. warm and dry), while ClimatePC2 was associated most strongly with seasonality. We used ClimatePC1 and ClimatePC2 to test hypotheses that climate of origin is associated with variation in both thermotolerance and Hsp101 expression.

4.2.8 Statistical analysis

One analysis was used for general characterization of responses to heat treatments and to test the hypothesis that populations differed in their responses to the heat treatments. The four heat treatments compose a factorial design in which there are two levels of acclimation (22°C control, or 38°C acclimation) and two levels of heat challenge (42°C or 45°C). We classified all seedlings as to population of origin, survival or death, and post-stress root growth. For all the tests of treatment and population effects on seedling survival described below, logistic regression analysis was applied using Proc GENMOD in SAS 9.1 (SAS Institute 2005). Our logistic regression model therefore predicted seedling survival as a function of population, acclimation, and heat challenge. Three population interaction effects were also included: population of origin

x acclimation treatment, population of origin x heat challenge, and acclimation x heat challenges as well as the three-way interaction of population of origin x acclimation treatment x heat challenge. Population, acclimation treatment, and heat challenge were all considered as fixed effects in this study. For testing heat treatment effects on post-stress root growth and Hsp101 expression, ANOVAs tested the effects of population, acclimation, heat challenge and their interactions in Proc GLM (SAS Institute 2005). In this case, we accounted for the effect of initial size on post-stress growth by using pre-stress root length as a covariate. Both pre- and post-stress root length were log-transformed, achieving very close fits to normal distributions of residuals. When interaction tests were clearly non-significant, the analysis was re-run without them. The full-model ANOVA was followed by separate tests by 42°C vs. 45°C heat challenge. For all three measures, analyses were further dissected to examine within treatment effect of population and its interactions (Table 24), however the power of the within treatment tests is relatively low. All means comparisons conducted in Proc GENMOND or Proc GLM were followed by Bonferonni critical p-value adjustment including Holm's correction (Holland and Copenhaver 1987).

We tested for a relationship between Hsp101 expression and thermotolerance by conducting two regressions. Each regression treated Hsp101 expression (log transformed) as the putative causal variable. One regression tested for Hsp101 expression effects on population mean percent survival, and one tested for Hsp101 expression effects on population mean post-stress root growth (log transformed). Data were pooled across treatment in these regressions. The regressions were conducted in Proc REG (SAS Institute 2005).

We further tested whether variation in thermotolerance and Hsp101 expression matched with variation in the climates of population origin. The climates of population origin,

quantified as ClimatePC1 and ClimatePC2 values, were used to examine the evolved effect of past climate on both the population mean thermotolerance measures (percent seedling survival and post-stress root growth) and on Hsp101 expression. Logistic regression was performed for testing the prediction of post-stress percent seedling survival with ClimatePC1 and ClimatePC2 using Proc GENMOD (SAS Institute 2005). Generalized linear regression using Proc GLM (SAS 2005) tested the prediction of past climate influences on post-stress root growth and Hsp101 expression separately. In all cases, the design effects (acclimation treatment and heat challenge) were treated as fixed effects. The full-model regressions were followed by separate tests of 42°C and 45°C heat challenge.

4.3 RESULTS

4.3.1 Thermotolerance phenotype depends on acclimation and heat challenge temperature

ANOVA provided the confidence behind the statements that follow in this paragraph; relevant here are the Heat Challenge and Acclimation*Heat Challenge rows in Table 4a, in conjunction with the associated means comparisons shown in Fig.10. These analyses indicate that A. thaliana accessions from NE Spain suffered reduced survival and root growth when exposed directly to 45°C (CT45 treatments), in keeping with prior experiments using standard laboratory lines of A. thaliana (eg. Queitsch 2000). Seedling survival and post-stress root growth differed between the 42°C and 45°C heat challenges (Fig.10). This difference in heat challenge effect is further separable by the presence or absence of the 38°C acclimation treatment. Without acclimation, a

45°C heat challenge (CT45) significantly decreased percent seedling survival and post-stress root growth compared with a 42°C heat challenge (BT42). However, with a 38°C acclimation, a 45°C heat challenge (AT45) had no discernable effect on seedling survival, but did show decreased root growth, both compared with the effects of a 42°C heat challenge (AT42). The effect of a 38°C acclimation treatment varies according to response trait and heat challenge. At 42oC, no effect of acclimation for was detected for either response traits (CT42 vs. AT42). However, at 45°C acclimation improved thermotolerance for both traits (CT45 vs. AT45). Prior acclimation at 45oC (AT45) significantly increased root growth by about 3-fold compared to CT45. However this amount of growth is still only about 50% of the total root length achieved in the CT42 treatment. Thus at 45°C the induction of AT mechanisms is sufficient to insure high survival, but not sufficient for rapid post-stress growth recovery.

4.3.2 Hsp101 expression varies with acclimation and heat challenge temperature

Hsp101 expression was significantly up-regulated in acclimated plants that were later exposed to both 42°C and 45°C (Fig.11, Fig.12, Table 4 $p<0.0001$). The content of Hsp101 as a result of acclimation at AT42 and AT45 was indistinguishable (Fig. 11, Fig.12). The heat challenges also led to an increase in Hsp101 expression at both CT42 and CT45 (i.e. without prior acclimation). However, the amount of Hsp101 expressed at CT42 and CT45 was significantly less than that observed at AT42 and AT45: 34% and 85% less Hsp101 at CT42 (Table 4b, Fig.11, $p=0.0004$) and CT45 respectively (Table 4c, Fig.11, $p<0.0001$). This difference in Hsp101 expression between the two CT treatments may be caused by strongly compromised cellular function at 45°C and only partially compromised cellular function at 42°C. Yet even this reduced expression level at 42°C was sufficient to enhance survival and post-stress root growth to an extent that was

indistinguishable from that in seedlings in the AT42 treatment. However, the same was not true for CT45 plants; those plants had greatly reduced Hsp101 expression (85% less) compared to AT45 plants, average seedling survival rates of only 40%, and post-stress root growth of only about 20% of that observed at AT45. Our interpretation is that 42°C heat challenge is mild enough that many plants can maintain sufficient cellular function to rapidly induce acquired thermotolerance mechanisms, including Hsps. In contrast, at 45°C cellular function appears to be compromised so quickly that induction of thermotolerance mechanisms is substantially impaired and the seedlings cannot protect cellular function from further damage. The differences among populations that we see in Hsp101 accumulation in acquired thermotolerance may be due in part to evolved differences in the maximum level accumulation of Hsp101. It may also be due to the differential response to 38oC among these populations, that is, some populations may produce substantially more Hsp101 while some may produce less at 38 oC. Although we don't have a way to address this, the difference in Hsp101 response to the 38oC acclimation temperature is very unlikely to contribute to the difference we see. Additionally, 42°C might not represent a significant stress, while 45°C represent a more stressful temperature.

4.3.3 Populations of A. thaliana vary in their responses to high temperature

Overall, we observed genetically-based variation among populations in thermotolerance (seedling survival: $p=0.0007$; post-stress root growth: $p<0.0001$; Table 2a) and Hsp101 expression ($p=0.02$; Table 4a). Within heat treatments populations differ significantly except in seedling survival at CT42 [see Table 24]. Populations significantly vary in post-stress root growth within all four treatments [see Table 24]. Moreover, populations also differ significantly in their responses to the two heat challenges, as reflected in the Population*Heat challenge

interaction (Table 4a), indicating complex among-population variation in stress dependent responses.

Significant variation in Hsp101 expression was observed among populations when all the data were pooled (Table 4a; Fig. 12). However, the inherent variability in Hsp101 expression and in quantification from western blots, together with limited sample number, resulted in low power to detect differences among populations when treatments were analyzed separately [see Table C1]. Thus in further analyses and discussion, we focus on the general relationship with the pooled data between Hsp101 expression and the thermotolerance measures at the population level.

4.3.4 Variation in Hsp101 accumulation is positively associated with variation in thermotolerance

At 45°C, higher Hsp101 accumulation was associated with higher percent seedling survival, explaining 37% of the variation (Fig. 13a; p=0.0003). Similarly, higher Hsp101 accumulation was associated with increased post-stress root growth, accounting for 15% of the variation (Fig.13b; p=0.04). Plants with low Hsp101 accumulation exhibited very low rates of survival and below average rates of post-stress root growth. In contrast, Fig.4 also shows that plants with high Hsp101 accumulation range from very low to very high survival and post-stress root growth. This pattern demonstrates that Hsp101 up-regulation is necessary but not sufficient to ensure high levels of thermotolerance.

It is important to note that many other Hsps are co-regulated with Hsp101. Thus we cannot ascribe a direct causal relationship to the significant regression of survival or post-stress

growth on Hsp101 content. In fact, it is most likely that thermotolerance is conferred by a whole suite of genes, with Hsp101 playing a direct and essential role (Hong et al. 2003).

4.3.5 The observed variation in thermotolerance and Hsp101 expression appears to be adaptive

Both thermotolerance measures and Hsp101 accumlation co-varied with ClimatePC1 of the populations' sites of origin under some but not all treatments (Table 5). Overall, ClimatePC1 significantly predicted seedling survival (Table 5a; $p = 0.04$). That is, populations from the cooler, moister end of the climate gradient exhibited increased percent seedling survival compared to populations from hotter and drier climates. This relationship was also significant at the 45°C heat challenge (Table 5c; $p=0.008$) and this effect at 45°C appears to have driven the significance of the analysis overall. Regression of post-stress root growth on climatePC1 was not significant overall (Table 5a). However, for plants exposed to 45°C, post-stress root growth increased significantly with ClimatePC1 (i.e. increased toward the cooler and moister end of the climate gradient compared with the hotter and drier end (Table 5c; $p=0.0003$). Hsp101 expression was not significantly associated with climatePC1 overall (Table 5a), but was positively associated with climate PC1 at 42°C (Table 5b; $p = 0.01$). ClimatePC2 was not a significant predictor of thermotolerance measures or Hsp101 accumlation in either the full model or in the separate treatment analyses (Table 5).

4.4 DISCUSSION

This study demonstrated a general pattern among plant populations of natural variation in thermotolerance, here measured by seeding survival and post-stress root growth, and accompanied by increases in accumulation of heat shock protein. Furthermore, the pattern of variation in thermotolerance is associated with variation in the climates of the populations' sites in northeastern Spain. We found that pre-exposure to mild heat stress resulted in acquired thermotolerance and an associated up-regulation of Hsp101. The acclimation treatment increased thermotolerance substantially. Hsp101 expression also varies among populations and shows the first evidence of genetically-based variation in expression associated with climate.

It may seem counterintuitive that the genetic lines from cooler, moister end of the climate gradient exhibited higher seedling survival and post-stress root growth than lines from the hotter, dryer end of the gradient. The lines from the cooler end of the gradient also showed greater accumulation of Hsp101. Importantly, these results are concordant with results of a previous study of geographic variation in Hsp101 expression (Tonsor *et al*. 2008). Genetic lines of *A. thaliana* from cooler, moister, northern latitudes exhibit greater Hsp101 expression than lines from the southern limits of the species' range (Tonsor *et al.* 2008). Populations of *Arabidopsis* appear to be differentiated in their mechanisms of response to heat stress with more southern populations relying less on acquired thermotolerance. Previous studies in the same populations used here suggest that low elevation populations may instead avoid heat by maturing earlier (Wolfe and Tonsor 2014; Montesinos-Navarro *et al.* 2012). A similar pattern, that populations from cooler environments had higher induced/acquired thermotolerance, was also found by Barua *et al.* 2008. A number of other factors may also contribute to the seemly counter-intuitive results. Firstly, populations from higher altitudes and cooler climate are exposed to more variable

environments - higher range of temperatures, such as high variability in annual or diurnal temperature. Barua *et al.* (2008) further found that variation in induced tolerance and Hsps was related to temperature variability, in that populations that are more likely to experience multiple heat stresses had higher tolerance and Hsp accumulation. Further analysis on the micro-climate variability will provide more information on these populations' local climate conditions. Secondly, plants from lower altitudes and warmer climate may rely on basal mechanisms of thermotolerance that are thought to be lower cost compared to induced mechanisms. Thirdly, plants from lower altitudes and warmer climate may simply avoid heat stress by maturing earlier. Finally, plants from high altitudes are faced with other stresses like UV-B radiation which may be correlated with thermotolerance and Hsps. However, our populations are not sufficiently high in altitude that annual mean UV is meaningfully greater along the elevation gradient (data extracted from DIVA-GIS 7.5.0).

The seeds used in this experiment are descended from field-collected genotypes through at least two, most of them three or four, generations of single seed descent in growth chambers. Within the growth chambers individual seed plants were randomized in their locations. We can therefore be confident that the differences observed among populations reflect differences in population genetic composition rather than uncontrolled environmental differences during the experiment or environment maternal effects carried over from the field. Picó *et al.* (2008) demonstrated that the populations from this region of Spain share a common ancestry, no doubt resulting from their ancestor's emergence from their ice age refugium and their subsequent spread across the landscape of northeastern Spain (Picó *et al.* 2008). The significant association of seedling survival rates with the first principal component of climate variation (ClimatePC1) suggests the signature of adaptation to the specific habitats from which the populations were

collected. That we only detect an association of variation in seedling survival, post-heat root growth, or Hsp101 expression with climate of origin under some circumstances suggests three additional explanations for the observed population variation. First, the power of this experiment may not have been sufficient given the inherent variability in seedling root growth and Hsp101 quantification in our experimental design and execution. Second, macro-scale climate may not be the only factor involved in adaptation. In the highly dissected topography of northeastern Spain many unmeasured site characteristics can influence microclimate, including slope, aspect, height of surrounding vegetation, and thermal properties of the substrate. All of these could either directly influence the temperatures experienced, or influence the trade-offs involved in implementing various potential thermotolerance mechanisms. Finally, founder effect and random genetic drift may account for some of the observed variation. However, the adaptive clines observed in a great many traits previously examined in these populations make it unlikely that random genetic drift is the predominant cause of the observed variation (Montesinos-Navarro *et al.* 2011; Montesinos-Navarro *et al.* 2012; Wolfe and Tonsor 2014).

Nearly all of the existing knowledge of thermotolerance and heat shock response in *A. thaliana* has been gained from studies of seedlings. It is important to note that these studies cannot fully put cellular mechanisms of thermotolerance into the context of mechanisms of thermotolerance relevant in field settings. First, the seedling stage is the least likely stage to experience lethally high temperatures. *Arabidopsis thaliana* seeds germinate under the most benign conditions experienced by the plant- the cool, moist periods of late fall and early spring. Second, like most plants, *A. thaliana* possesses a number of mechanisms for avoiding high temperatures that cannot be used at the seedling stage because the necessary structures and functions simply are not present. These include avoidance of periods of high temperature

through the evolution of altered life history timing (Montesinos-Navarro *et al.* 2012), variation in constitutive high leaf angle, leaf hyponasty and petiole elongation in response to elevated temperature (Polko *et al.* 2010; Gray *et al.* 1998), and transpirational cooling. Full understanding of high temperature responses and their consequences will require integrated study of cellular, physiological and developmental responses throughout the plant life cycle.

Table 3. Population locations and climate principle components.

Population	Longitude	Latitude	Elevation (m.a.s.l.)	ClimatePC1	ClimatePC2
PIN	2.6591	41.6592	109	-3.6458	-1.7597
RAB	3.0537	42.3781	110	-4.4803	0.8848
SPE	2.9162	41.9275	332	-2.9213	-1.6334
BAR	2.1278	41.4322	340	-4.58	-1.1973
HOR	2.6202	41.6645	351	-2.7083	-1.8691
ARB	2.4941	41.8143	440	-2.1884	-0.6771
COC	3.1653	42.3263	519	-3.2761	-0.087
POB	1.0235	41.3526	597	-2.0028	3.7342
BOS	0.6913	42.7836	719	2.6056	2.6257
MUR	2.0002	41.6757	836	-2.0033	0.2208
VDM	1.0249	42.0292	912	1.2933	2.6184
ALE	1.3187	42.4105	1163	2.3143	1.2497
BIS	0.5334	42.4878	1397	5.8196	-2.246
PAL	1.2926	42.4312	1491	4.2579	0.6184
VIE	0.7606	42.6256	1538	5.7257	-1.1941
PAN	-0.231	42.762	1664	5.7899	-1.2883

*Condensed from Table S1 and Table S3 in Wolfe and Tonsor (2014).

Table 4. ANOVA table for seedling survival, post-stress root growth and Hsp101 expression in full model and separate analysis by heat challenge temperature. Post-stress root growth was adjusted using pre-stress root growth as a covariate ($p<0.0001$). -------: non-applicable. All models exhibit overall significance. When interaction terms were non-significant the model was run again without the non-significant interactions. All reported p-values are from the re-analysis.

Source	Seedling survival	Post-stress root growth	Hsp101 expression
a. Full Model			
Model r^2	——	0.89	0.50
Population	0.0007	<0.0001	0.02
Acclimation	<.0001	0.002	<0.0001
Heat challenge	0.08	<0.0001	<0.0001
Acclimation*Heat challenge	0.0006	0.03	<0.0001
Population*Acclimation	NS	0.09	NS
Population*Heat challenge	0.009	<0.0001	NS
Population*Acclimation*Heat challenge	NS	0.0002	NS
b.42 °C heat stress			
Model r^2	——	0.89	0.36
Population	0.01	<0.0001	0.03
Acclimation	NS	NS	0.0004
Population*Acclimation	NS	0.005	NS
c. 45 °C heat stress			
Model r^2	——	0.94	0.60
Population	0.0005	<0.0001	NS
Acclimation	<.0001	<0.0001	<0.0001
Population*Acclimation	<.0001	0.0002	NS

Table 5. ANOVA table for seedling survival, post-stress root growth and Hsp101 expression with ClimatePCs in full model and in separate analyses by heat challenge temperature. Post-stress root growth was adjusted using pre-stress root growth as a covariate ($p<0.0001$). -------: non-applicable. All models are significant. All reported p-values are from a re-analyzed model after removing the non-significant interaction terms (NS).

Source	Seedling survival	Post-stress root growth	Hsp101 expression
a. Full Model			
Model r^2	------	0.86	0.41
ClimatePC1	0.04	NS	NS
ClimatePC2	NS	NS	NS
Acclimation	<0.0001	0.001	<0.0001
Heat challenge	0.09	<0.0001	<0.0001
Acclimation*Heat challenge	0.001	0.04	<0.0001
b.42 °C heat stress			
Model r^2	------	0.83	0.17
ClimatePC1	NS	NS	0.01
ClimatePC2	NS	NS	NS
Acclimation	NS	NS	0.001
c. 45 °C heat stress			
Model r^2	------	0.92	0.49
ClimatePC1	0.008	0.0003	NS
ClimatePC2	NS	NS	NS
Acclimation	<0.0001	<0.0001	<.0001
ClimatePC1*Acclimation	0.02	NS	NS

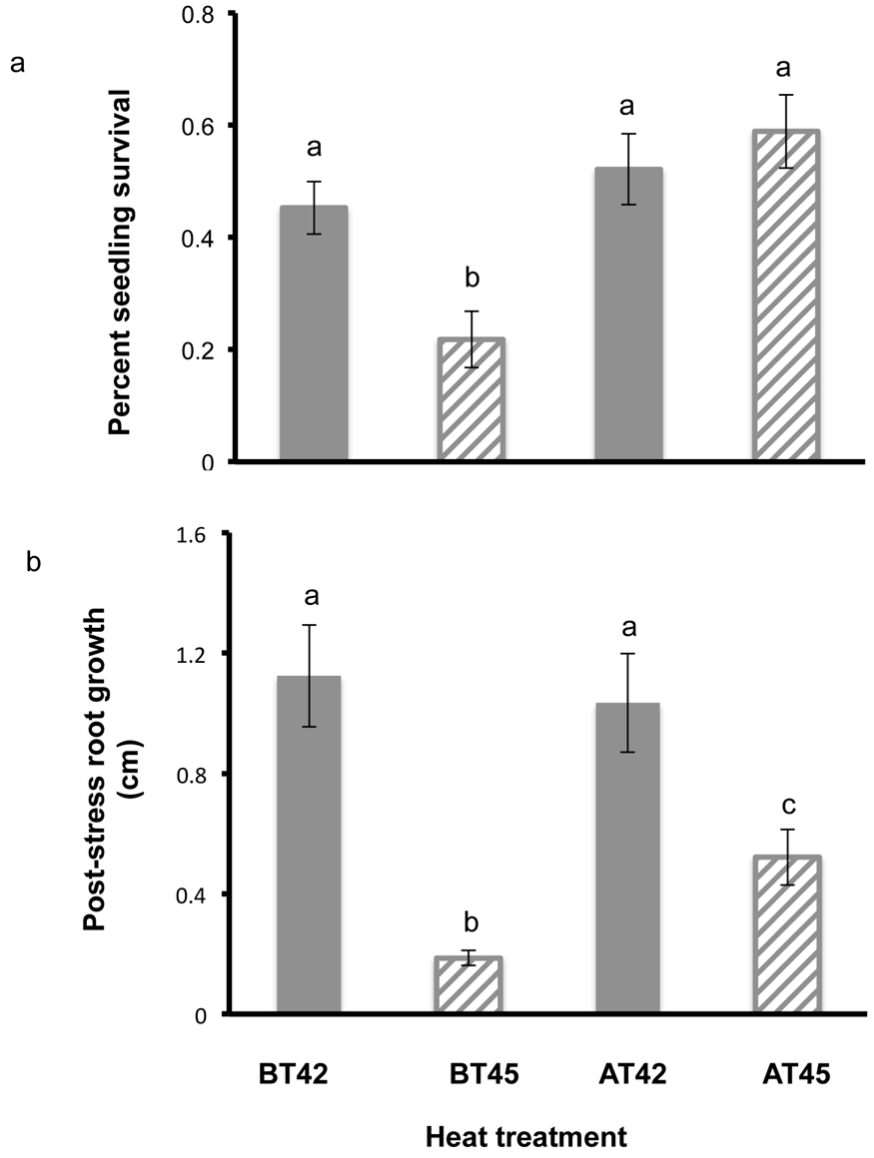


Figure 10. (a) Percent seedling survival and (b) post-stress root growth one week after heat stress, by heat treatment. For percent seedling survival (a), data displayed are treatment means based on population survival percentage within treatment. For post-stress root growth (b), data displayed are means of individuals within treatments. Vertical lines represent standard errors. Treatments with different letters above the bar are significantly different with an initial rejection criterion of $p < 0.05$ adjusted for multiple comparisons using the sequential Bonferroni method.

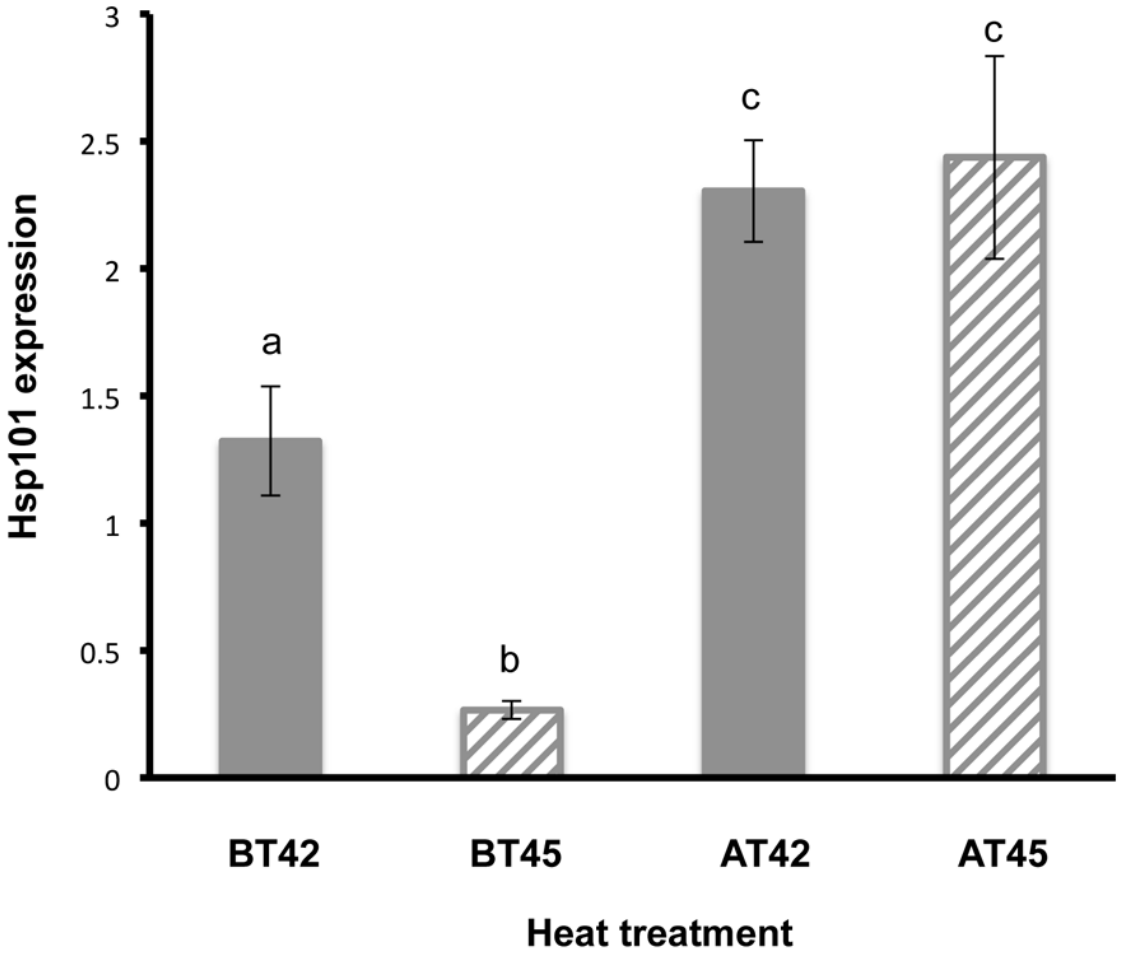


Figure 11. Hsp101 expression by heat treatment. Data displayed are means of individuals within treatments (vertical lines represent standard errors). Hsp101 expression is adjusted to a bulk Hsp101 expression standard to provide a standardized relative comparison among samples. Treatments with different letters above the bar are significantly different with an initial rejection criterion of $p < 0.05$ adjusted for multiple comparisons using the sequential Bonferroni method.

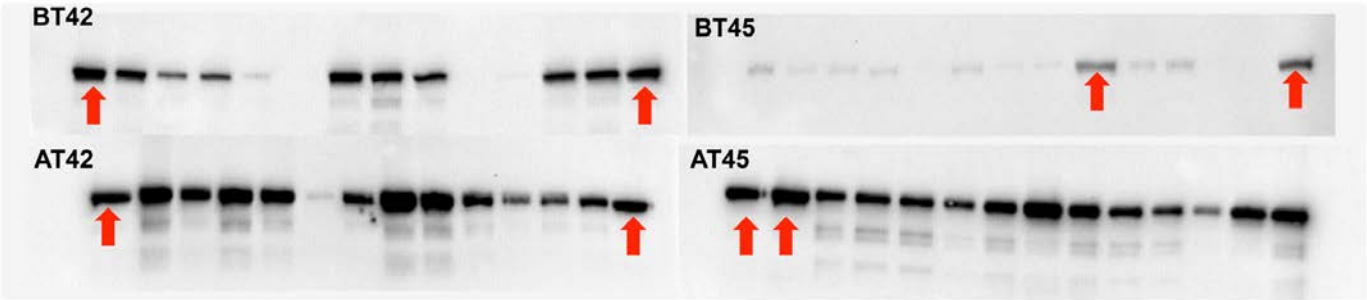


Figure 12. Western blots showing differences in Hsp101 expression among treatments. Variation within treatments is due to significant population variation in expression as well as sample variation in total protein content. A molecular ladder was used in each gel to identify Hsp101 position (ladder not visible in images). Within treatment, genotypes within populations were randomly assigned to the lanes of western blots. For analysis, variation in the amount of protein loaded was used to adjust Hsp101 expression per unit protein. A bulk sample was created and loaded in two lanes on each gel as a control for gel-level variation. Arrows indicate the bulk controls in each western blot.

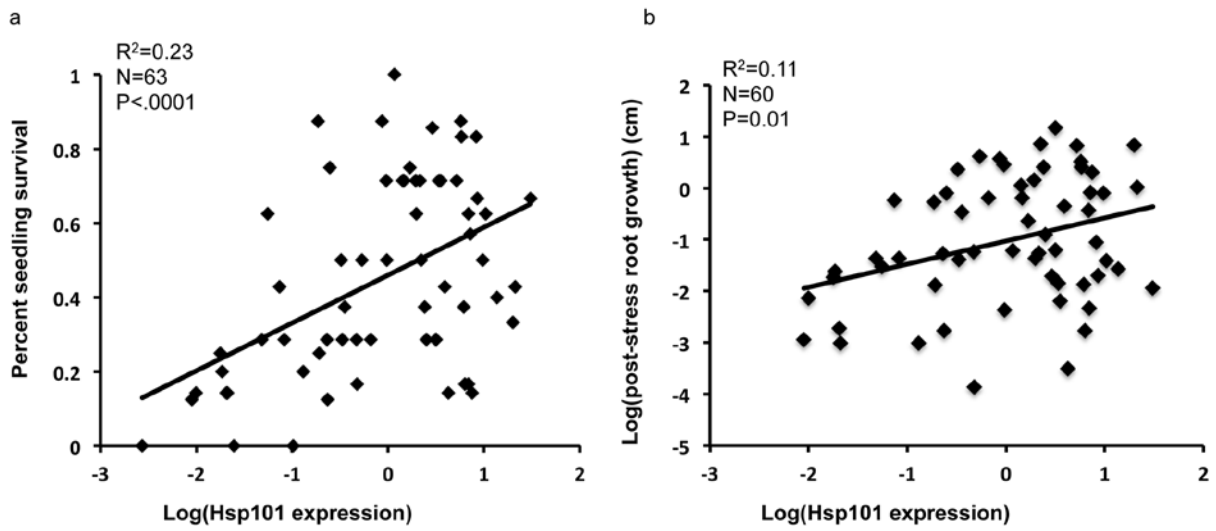


Figure 13. Regression of (a) percent seedling survival and (b) post-stress root growth (cm, log transformed) on Hsp101 expression (log transformed) at 45ºC heat challenge (BT45 and AT45). Data displayed are population means of each treatment for all four treatments. a) Percent seedling survival = 0.47 + 0.16 * log (Hsp101 expression); b) Log (post-stress root growth) = -1.50 + 0.32 * log (Hsp101 expression). Triangle: BT45; Circle: AT45. The colors of the populations were arranged along a red-green-blue gradient, from low to high elevation, e.g. red represents low elevation populations while blue represents high elevation populations.

5.0 CONTRASTING AVOIDANCE–TOLERANCE IN HEAT STRESS RESPONSE FROM THERMALLY CONTRASTING CLIMATES IN *ARABIDOPSIS THALIANA*

5.1 INTRODUCTION

Abiotic stresses, such as temperature and drought, are main range limitation determinants. Heat stress imposed by daily temperature fluctuation can cause severe damage to plants, including reduction of plant growth and alterations in photosynthesis and phenology. Such disruptions are ultimately likely to cause reduction in resources available for reproduction(Hasanuzzaman et al. 2013). It is therefore quite likely that frequent heat stress will reorganize allocation and physiology through selection for the highest fitness response to high temperature events. Careful observation of the relationship between a plant's thermal environment and the specific mechanisms of adaptation to heat stress in wild populations is very limited, despite its likely relationship to extinction at the warmer end of species' ranges.

In general, we define a heat stress as a diurnal temperature pattern in which plants display reduced fitness compared to some other temperature pattern (SØrensen 2001). Usually, the maximum stress temperature is about 10 to 15^{o}C higher than the optimum for broadly distributed species and as little as 5^{o}C higher than the optimum in species with narrow geographic ranges (Lindquist 1986).

Plants have developed both long-term and short-term adaptations to high temperature (Hong et al. 2003). Evolutionary adjustments of the timing of life history events (Montesinos-Navarro et al. 2011) and further adjustments in the timing of allocating of resources to rosette and inflorescence (Wolfe and Tonsor 2014), are mechanisms that allow annual plants to escape from the most stressfully high temperature period by adjusting life cycle timing. To further reduce or prevent stress from high temperature during the active growing season, adaptive responses can be described as part of two main strategies, avoidance and tolerance (Sakai and Larcher 1987).

Stress avoidance is a strategy through which plants adjust their internal states in ways that reduce exposure to a potentially damaging environment (Touchette et al. 2009, Puijalon et al. 2011). For most plants, leaves are the most important structure for obtaining energy and carbon (but see (Earley et al. 2009)). On average, avoidance can lower leaf surface temperature across growing season by 4^{o}C compared to ambient temperature in cotton (Wiegand and Namken 1966). Generally, the higher the air temperature, the larger the differential between air and leaf can be (Linacre 1967, Wilson et al. 1987). Leaf temperature thus becomes an ideal indicator to keep track of plants' heat avoidance.

Avoidance can also be achieved by leaf orientation adjustment (Jones and Corlett 1992, Zlatev et al. 2006). Many plants adjust leaf angle, thus reducing the leaf area that is exposed to heat from sunlight (Bradshaw 1972, Huey 2002). Populations originating from high temperature sites have higher leaf angle in heat stress in *Arabidopsis thaliana* (Vile *et al.* 2012) and in *Aractostaphylos* species (Shaver 1978, Ehleringer 1987, Fu 1989).

Avoidance can also be achieved through transpiration which is immediately elevated at high temperature, thus cooling the leaf surface temperature (Shah et al. 2011). Also, the

threshold temperature that controls the relative rate of transpiration is species-specific (Mahan 1990). The transpiration process is closely connected with stomatal opening (Burke and Upchurch 1989). One potential constraint on transpiration-driven heat stress avoidance is that high temperature often co-varies with a dry environment in nature. Plants from drier and warmer sites show higher water use efficiency, compensating for large water losses due to transpirational cooling in *Boechera holboellii* populations (Knight et al. 2006). However, phylogenetic analysis of 28 dominant species in a Mexican evergreen shrubland also showed a correlation between steeper leaf angle and low transpiration rate as an adaptation to dry climate (Falster and Westoby 2003, Valiente-Banuet et al. 2010). These contrasting selection pressures on transpiration in combined heat and drought stress further complicate the evolution of avoidance in nature (Vile et al. 2012).

When internal temperatures rise sufficiently despite any avoidance mechanisms possessed by the plant, heat stress can damage cells in a variety of ways. High cellular temperature affects both cellular structural integrity and protein function, causing membrane disruption as well as disruption of metabolic function through production of reactive oxygen species (ROS) (Schöffl et al. 1998) and enzyme denaturation (Blum and Ebercon 1981, Reynolds et al. 1994, Ismail and Hall 1999). Photosynthesis is especially heat-sensitive (Berry and Bjorkman 1980, Reynolds et al. 1994, Sharkey et al. 2008). In plants measurement of extent to which photosynthetic rate is depressed can be an effective measure of functional disruption. Eventually, as temperatures rise damage is sufficient to affect a plant's survivorship and fecundity (Senthil-Kumar et al. 2007).

Heat tolerance is the ability of plants to minimize or repair damage while experiencing a high internal temperature (Touchette et al. 2009, Puijalon et al. 2011). Heat tolerance

mechanisms include protection and repair of damaged cell structures, structural proteins, and enzymes (Shah et al. 2011). While heat tolerance is complex (Kotak et al. 2007) and incompletely understood, it is known to involve up-regulation of two classes of molecules: heat shock proteins (thereafter, Hsps) (Queitsch 2000b, Hong and Vierling 2001, Wang et al. 2004) and plant hormones (Larkindale 2004, He et al. 2005, Larkindale and Huang 2005). Hsps are a group of molecular chaperones involved in dissolving and refolding aggregated cellular proteins, under both normal and stressful conditions (Hartl 1996). Members of the Hsp100/ClpB family have shown a significant role in heat tolerance in *Saccharomyces cerevisiae* (Hsp104) (Sanchez and Lindquist 1990) and *Arabidopsis thaliana* (Hsp101) (Queitsch 2000a, Hong and Vierling 2001). Plant hormones, especially salicylic acid (SA), are a common stress response. SA reduces reactive oxygen species (ROS) accumulation and affects a great many other processes in the plant. SA expression modulation and its effects are not fully understood, but SA's importance for a variety of stress responses is well documented (Delaney et al. 1994, Klessig and Malamy 1994, Clarke et al. 2004, Yuan and Lin 2008, Vlot et al. 2009).

Plants in environments with frequent heat stress tend to evolve greater tolerance (Huey 2002), but how avoidance varies in adaptation to heat stress remains unclear. Avoidance and tolerance can have different costs and benefits depending on complex aspects of the growth environment. Thus different environments may favor tolerance or avoidance in response to the balance of selection acting on the mechanisms involved in each strategy (see for example for drought tolerance / herbivory avoidance (Siemens and Haugen 2013), for irradiance and water availability (Sánchez Gómez et al. 2006). It is however a mystery how avoidance and tolerance work together to contribute at the whole plant fitness level. The question of the potential relationship between avoidance and tolerance, different strategies responding to the same stimuli,

is of primary ecological interest, as it may reveal constraints that limit the evolution of the traits involved in the response and variation in a broad range of architectural traits, all involved in these strategies.

In this study we use a set of wild-collected *Arabidopsis thaliana* populations that exhibit clines in many traits in association with a climate gradient (Montesinos-Navarro et al. 2009, Montesinos-Navarro et al. 2011, Wolfe and Tonsor 2014). Previous work indicates that the climate gradient includes gradients in both temperature and precipitation. Thus we are particularly interested in heat stress in the context of water use.

We ask two questions. Do populations from thermally contrasting climates:

1) Display the same reductions in fitness with heat stress?
2) Exhibit contrasting avoidance and tolerance strategies?

Four low- and four high- elevation populations, each with four genotypes, were collected from northeastern Spain. In this area, climate is highly correlated with elevation (Montesinos-Navarro et al. 2009, Wolfe and Tonsor 2014). Low elevation populations experience hotter and dryer conditions, while high elevation populations experience colder and wetter conditions. In this study, we first looked at the fitness effect of repeated heat stress episodes for all the plants. We then compared avoidance and tolerance strategies in heat stress response in plants from the contrasting climates. We further explored possible causally-connected traits for both avoidance and tolerance. For avoidance, we looked at rosette angle and transpiration rate. For tolerance, we looked at the accumulation of Hsp101 and SA.

5.2 MATERIALS AND METHODS

5.2.1 Materials and heat treatment

Plant lineages were collected from NE Spain as seeds (Montesinos-Navarro et al. 2009, Montesinos-Navarro et al. 2011, Montesinos-Navarro et al. 2012, Wolfe and Tonsor 2014) and grown for at least three generations in common controlled environmental conditions to remove any maternal environmental variance that might otherwise have carried over from the field. The geographic locations of these populations can be seen in Fig.38 (adapted from Fig.1 in Montesinos-Navarro et al. 2001). Seeds were germinated and maintained at 22°C for 3 weeks (16 hrs light/8 hrs dark, 200 μM $m^{-2}s^{-1}$) after 5-day stratification at 5°C in the dark. Since these populations are more likely to experience heat stress at the bolting stage in nature, heat treatments were performed at bolting stage (stage 6 - 6.10 based on Table 1 in Boyes et al. 2001). Seedlings therefore experienced a 4-week vernalization at 5°C (10 hrs light / 14 hrs dark, 150 μM $m^{-2}s^{-1}$), to synchronize flowering time. After return to control growth conditions (16 hrs light/8 hrs dark, 200 μM $m^{-2}s^{-1}$), plants were checked every day and those at the bolting stage were transferred to a separate chamber for the heat treatment. Growth, control, and heat treatment were all conducted in our Conviron PGW36 controlled environment growth chambers (http://www.conviron.com) at the University of Pittsburgh. Plants from each population were blindly partitioned into two groups and randomly ordered across populations in each group. One group was the control group, in which plants were maintained at 22°C all the time, the other was the heat treatment group, in which chamber air temperature was increased steadily over 15 minutes to reach 45°C, maintained at 45°C for 3 hrs and then brought back to 22°C. This treatment was repeated twice a week from each plant's first heat treatment till harvest (following

method of Larkindale & Vierling 2008). We harvested all the plants 60 days after their first heat treatment. Avoidance and tolerance measures were performed during the first heat treatment, while fitness was estimated at harvest.

5.2.2 Fitness quantification

We were able to measure total plant fitness 60 days after the first heat treatment, since plants were at that time nearly completely senesced. As explained in the results, there were two distinct types of fruits, aborted and mature. The distinction between the aborted and mature was visually obvious. Sampling a substantial number of aborted fruits showed that they contained no viable seeds. Only mature fruits were included in the fitness quantification. The length of the fruit in *Arabidopsis* is highly correlated with the number of seeds within the fruit (Alonso-Blanco et al. 1999). Thus we used summed fruit length as a measure of fitness. We measured the length of five randomly chosen normal and mature fruits, two from the main (apical meristem) stem and three from secondary (lateral meristem) stems, to estimate the average fruit length. We then counted the total fruit number for each plant. Fitness, here summed fruit length, is equal to the fruit number times the average fruit length, similar to Wolfe & Tonsor (2014).

5.2.3 Resource allocation quantification

We anticipated that heat stress would change resource allocation to the reproductive system and re-shape the reproductive structures, reflected in changes in reproductive branch lengths, number of branches and dry mass. To assess the potential change in resource allocation to reproduction, we partitioned the plants into rosettes, inflorescences and roots, dried them at 65°C for at least

three days, and recorded the dry mass of each component. Prior to drying we also measured the length of all the reproductive portions of inflorescence branches (length from insertion of the lowest fruit to the apex) with a Map Wheel (scalex.com) and counted the number of basal branches in each plant.

5.2.4 Avoidance characterization

A direct metric to indicate the level of avoidance is the difference between rosette temperature and air temperature. For rosette temperature, a thermocouple was placed in the center of the rosette, not touching the rosette surface, 15mins after the heat stress initiation. For air temperature, a thermocouple was suspended at height of the apical meristem of the tallest inflorescence in free air. For each plant the difference between rosette temperature and air temperature was calculated as the difference between the temperatures of these two thermocouples. To better understand the physiological basis for variation in rosette temperature among populations, rosette angle and transpiration rate were also quantified. To measure leaf angle, the whole rosette was photographed from four vantage points 90 degrees apart. The rosette angle is the angle of a plant's most recently fully developed leaf to the horizontal line and was measured in ImageJ in each image (Schneider et al. 2012). We then averaged the four angles as a measure of rosette angle for each plant. We simultaneously measured transpiration and photosynthetic rate using a LiCor 6400XT gas exchange analyzer. A custom-made *Arabidopsis* single-leaf cuvette was used and one most recently fully expanded leaf was held in the cuvette until gas exchange became steady (see Fig.39). Five measurements of carbon assimilation and transpiration were recorded at 12-second intervals and the measures were then averaged. Immediately following gas exchange measurement, each leaf was imaged and leaf area was

calculated in ImageJ (Schneider et al. 2012). The photosynthetic and transpiration rates were calculated as rate per area.

5.2.5 Tolerance characterization

We use relative photosynthetic rate compared with control to quantify heat tolerance. Photosynthetic rate was measured as described above with the LiCor 6400XT gas exchange analyzer. To determine the relationship of Hsp101 and SA accumulation to tolerance, we collected leaf samples and quantified Hsp101 and SA immediately following heat treatment. Two newly fully developed leaves, one for Hsp101 and the other for SA quantification, were collected right after heat treatment, freeze dried and weighed. Hsp101 quantification was measured via western blot as described in Tonsor *et al.* 2008. SA was quantified with HPLC as described in Zhang et al. 2015.

5.2.6 Statistical analysis

For each measure, we performed a separate ANOVA analysis to look at whether the measure showed significant difference at the level of elevation group, population nested within elevation group, heat treatment and the elevation group * heat treatment interaction, using Proc GLM in SAS (SAS Institute 2005). Elevation and population were treated as fixed effects.

To assess the relationship between these potential covariates and our avoidance and tolerance measures, a multivariate analysis of variance (MANOVA) was used both for avoidance and tolerance in Proc GLM (SAS Institute 2005). For avoidance, the difference between air temperature and rosette temperature, hereafter DeltaT, was treated as the dependent variable,

with rosette angle, transpiration rate, heat treatment, elevation group and the elevation group * heat treatment interaction as independent variables. For tolerance, photosynthetic rate was treated as the dependent variable, while Hsp101 accumulation, SA accumulation, heat treatment, elevation group and the elevation group * heat treatment interaction were treated as independent variables. The nested effects, population nested within elevation group, genotype nested within population, were also included in the MANOVA for both avoidance and tolerance analyses.

To further explore the direct relationship between transpiration rate and avoidance we performed univariate regression of DeltaT on transpiration rate. Likewise we tested for relationships between Hsp101/SA and tolerance by regressing photosynthetic rate on Hsp101 or SA the independent variables, using Proc REG (SAS Institute 2005).

5.3 RESULTS

5.3.1 Heat stress caused reproductive disruption and fitness reduction

Two types of heat stress disruptions of reproduction were observed. First, in some cases heat stress damaged the apical meristem of flowering stems (Fig. 40). After the death of the apical meristem, additional secondary stems were initiated. Second, even if the apical meristem survived, heat nevertheless often led to failure of fertilization or early fruit abortion for flowers with active gametes at the time of the heat stress (Fig. 41). After heat stress, the apical meristem recovered growth but the fruits from the damaged portion of apical meristem did not successfully mature. Dissection of fruits from damaged apical meristem showed no viable seeds. We did not count such fruit in our measure of total fruit lengths.

Plants from low elevation produced greater total fruit length than plants from high elevation ($p<0.0001$) across both heat stress and control treatments. Across the experiment as a whole, plants from low elevation produced more basal branches ($p<0.0001$), greater reproductive length ($p<0.0001$), and lower root dry mass ($p<0.0001$) than plants from high elevation. Plants from both low and high elevations showed 15% reduction in total fruit length when exposed repeatedly to 45°C ($p=0.0007$, Fig.14a). Under heat stress, all populations showed about 25% longer reproductive length ($p=0.01$, Fig.14c), while only high elevation populations showed 23% more basal branches ($p=0.005$, Fig.14b) and 12% less root mass ($p=0.0001$, Fig.14d). We did not see a significant difference in the above responses to heat stress between low vs. high elevation populations (Table 25).

5.3.2 High elevation populations showed greater avoidance

All plants maintained rosette temperatures that were statistically significantly different from ambient air temperature under all conditions (Fig.15). The direction of the difference in rosette temperature depended on the ambient temperature. All plants, regardless of elevation of origin, increased their rosette temperature relative to ambient temperature under the 22°C control condition. High elevation populations increased about 1.2°C more than low elevation populations (high vs. low: 24.4°C vs. 23.2°C, $p<0.0001$).

When exposed to heat stress, however, both low and high elevation populations maintained rosette temperature significantly and substantially lower than ambient air temperature, on average by 7.7°C across all populations. High elevation populations reduced rosette temperature 1.8°C more than low elevation populations (low vs. high: 38.2°C vs. 36.4°C, $p = 0.004$). We saw greater heat stress avoidance in high elevation populations (Fig.15).

Considering both the control and heat stress treatments together, high elevation populations exhibit greater rosette temperature homeostasis than low elevation populations (Fig.15).

5.3.3 Low elevation populations showed greater tolerance

Heat tolerance is the ability of a plant to perform normal plant functions when exposed to high temperature. Here we measured photosynthesis, one of the key plant functions, as a measure of tolerance (Fig. 16). Greater photosynthetic rate indicates relatively higher tolerance. We saw a significantly lower photosynthetic rate in low compared to high elevation populations under the control temperature ($p<0.0001$).

However, with a 45^{o}C heat stress, low elevation populations showed no significant change in photosynthetic rate, while high elevation populations significantly reduced their photosynthetic rate (Elevation group*Heat treatment interaction: $p=0.003$, Table 25). Low elevation populations were significantly more heat tolerant than high elevation populations ($p<0.0001$).

5.3.4 Avoidance was positively associated with high transpiration rate and flat rosette angle

We measured rosette angle and transpiration rate as potential traits associated with avoidance (Fig. 17, Fig.18, Table 25).

Rosette angle differed between low and high elevation populations regardless of treatment, with low elevation populations exhibiting sharper rosette angle (low vs. high mean

rosette angle: p=0.0005). However, under our measurement protocol the rosette angle was not significantly affected by heat treatment (Fig. 17a).

High elevation populations showed significantly higher transpiration rate than low elevation populations under the control temperature ($p<0.0001$, Fig.17b). Transpiration rate was significantly increased with heat treatment ($p<0.0001$), with high elevation populations increasing significantly more than low elevation populations ($p=0.0002$).

Our MANOVA analysis explained 94% of the variation in avoidance ($p<0.0001$, Table 6). Significant interaction effects were observed between elevation groups and heat treatment ($p<0.0001$), indicating that low and high elevation populations have evolved different responses to heat. We also saw a significant effect of rosette angle ($p=0.005$). However, we did not see a significant effect of transpiration rate. We observed highly significant nested effects for both population and genotype ($p<0.0001$ for both).

To further explore the direct relationship between transpiration rate and avoidance, separate regression analyses in the two heat treatments of DeltaT on transpiration rate showed a significant positive relationship between transpiration rate and DeltaT at 45 ^{o}C ($p=0.009$, Fig.18), e.g., higher transpiration rate was associated with higher DeltaT. We did not see a significant relationship between transpiration rate and DeltaT in the control (Fig.18). Even though we did not detect a significant effect of transpiration rate on DeltaT in the MANOVA analysis, this separate analysis confirmed its direct effect on DeltaT at 45 ^{o}C. We consider these apparently contradictory results for transpiration rate's effects in the discussion.

5.3.5 Tolerance was negatively associated with Hsp101 and SA accumulation

With heat stress, Hsp101 accumulation was significantly increased 22 fold and 8 fold for low and high elevations populations, respectively (Fig. 19a, $p<0.0001$, Table 25). However, because of the large variation within each elevation group, we did not detect a significant difference between low vs. high elevation populations in Hsp101 accumulation in the heat treatment (Fig.19a).

Both free and total SA were higher in low elevation populations than in high elevation populations across the experiment as a whole (Fig. 19b, free salicylic acid: $p=0.0001$; total salicylic acid data not shown). However, with heat treatment, high elevation populations significantly increased free and total SA, while low elevation populations showed no significant difference compared to control (Fig. 19b) in the 45^{o}C heat stress.

Our MANOVA explained 93% of the variation in tolerance ($p<0.0001$, Table 7). Significant effects of Hsp101 accumulation ($p=0.005$), total SA ($p<0.0001$), free SA ($p=0.005$) were observed. The interaction between elevation group and heat treatment ($p=0.01$) was also significant (Table 7), indicating evolved differences in elevation groups in their response to high temperature.

Univariate regression analysis of photosynthetic rate on Hsp101 accumulation showed a significant negative relationship, e.g., higher Hsp101 accumulation was associated with lower photosynthetic rate ($p=0.03$, data not shown). A negative relationship was also found between photosynthetic rate and free SA accumulation ($p=0.005$). These two univariate analyses are concordant with the results of the MANOVA.

5.4 DISCUSSION

Here we have shown that a 45ºC repeated heat stress imposed periodically starting at the bolting stage is a significant heat stress for genetic lines collected from natural populations of *Arabidopsis thaliana* in NE Spain, since it caused significant decrease in fruit production compared to a benign control temperature (Fig.14). We then showed that, although both avoidance and tolerance were observable in all populations in response to heat stress, high elevation populations manifested more avoidance (Fig.15) and low elevation populations showed more tolerance (Fig.16). Our mechanistic analyses further showed that avoidance was positively associated with high transpiration rate and flat rosette angle (Fig.17, Fig.18, Table 6), while tolerance was negatively associated with Hsp101 and SA accumulation (Fig.19, Table 7). The 8 populations used in this study are part of 17 populations along a climate gradient described in previous studies. In those prior studies we observed strong clines in many traits (Montesinos-Navarro et al. 2009, Montesinos-Navarro et al. 2011, Montesinos-Navarro et al. 2012, Wolfe and Tonsor 2014). We previously showed that population genetic analyses strongly support the hypothesis that this cline results from local adaptation along a climate gradient associated with altitude (Montesinos et al. 2009, Montesinos-Navarro et al. 2011). Likewise the contrast in strategy in low vs. high elevation populations observed in this study indicates differential evolutionary responses to heat stress associated with adaptation to Mediterranean low elevation vs. interior high elevation climates.

Despite the importance of heat stress, there is very little work that examines genetically based adaptive differentiation among lineages in heat avoidance and heat tolerance. However, much work has been done on other abiotic stresses. Abiotic stresses, drought stress and salt stress in particular, show a similar pattern of avoidance vs. tolerance in various plant species. Farrant et

al. (1999) reported a negative relationship between drought avoidance and tolerance in three desiccation-tolerant angiosperm species (M.Farrant et al. 1999). Five herbaceous wetland plant species showed varying combinations of avoidance and tolerance in response to short-term drought stress (Touchette et al. 2007). Four of the species showed an avoidance strategy while all five species also showed a tolerance strategy. Rahman et al. (2011) compared the relative contribution of avoidance and tolerance to drought stress in two kiwifruit species, finding *Actinidia deliciosa* had lower avoidance and higher tolerance than *Actinidia chinensis* (Rahman et al. 2011). Similarly, Touchette et al. (2009) showed a contrasting response to salt stress in marsh halophytes *Juncus roemerianus* and *Spartina alterniflora*, in which *Juncus roemerianus*, experiencing transient salt stress exposure, showed salt avoidance and *Spartina alterniflora*, with frequent long-term salt exposure, showed salt tolerance (Touchette et al. 2009). A diverse array of abiotic stresses share some common pathways at both physiological and molecular levels (Pastori and Foyer 2002), suggesting that we might expect a similar pattern as we learn more from heat stress. Further exploration of the constraints on the evolution of the two strategies at morphological, physiological, genomic and gene expression level can provide insights in understanding the distribution pattern of plants and how adaptive responses evolve over time.

The contrast in evolved relative importance of avoidance and tolerance between our two climatic study regions indicates disruptive selection on heat stress response between the high and low elevation regions of our source populations. This disruptive selection, selecting for greater avoidance at high elevation but greater tolerance at low elevation, is likely the result of different costs for each strategy depending on the local physical environment.

In *Impatiens capensis* early season drought stress selects for avoidance but later drought stress favors tolerance (Heschel & Riginos 2005). *Nicotiana tabacum* shows a sequential

response in drought stress, first avoidance then tolerance, indicating avoidance is favored in short-term stress but tolerance is favored in long-term stress (Riga and Vartanian 1999). This is in accordance with our study in *Arabidopsis thaliana* as well. When the observed avoidance and tolerance patterns were put in the context of climate, we saw interesting associations of response to heat with climate of population origin. For example, we know that annual precipitation in high elevation is 550mm greater than precipitation in low elevation (Wolfe and Tonsor 2014) and continues longer into the summer season (Montesinos-Navarro et al. 2009). This may allow greater transpiration in high elevation populations, contributing to the greater ability to avoid high temperature we observed among high elevation populations. Studies on drought and salt stress also showed that plants that have greater access to water adopt avoidance rather than tolerance (Touchette et al. 2007, Touchette et al. 2009). Similarly, the average annual temperature is up to 11^{o}C higher in our low elevation sites compared to high elevation sites (Wolfe and Tonsor 2014); thus populations from low elevation are constantly exposed to higher temperatures compared to high elevation populations. This, combined with the lower availability of water for transpirative cooling, may explain why low elevation populations are more tolerant and less resistant than high elevation populations.

We found an increase in rosette temperature in the 22^{o}C control but a decrease in the 45^{o}C heat treatment compared to the ambient temperature for all populations. The difference between rosette temperature and air temperature is positive in cool but negative in hot air. The air temperature at which one observes zero air-leaf temperature differential has been called the "equality temperature" (Linacre 1964). The equality temperature is often around 30^{o}C in well-watered, thin-leaved plants (Linacre 1967) but is species-specific (Savvides et al. 2013). For example, cotton has an equality temperature of 27^{o}C (Upchurch and Mahan 1988). Based on our

data and assuming a linear response, we can draw approximate equality temperatures at 25.4^{o}C and 27^{o}C for low elevation and high elevation populations, respectively. This 1.6^{o}C difference in equality temperature reflects an intraspecific differentiation in homeostatic control in natural *Arabidopsis* populations. In a range of 22 – 45^{o}C, high elevation populations were more homeostatic than low elevation populations (Fig.15). Our study also supported (Mahan and Upchurch 1988) proposal that plants are capable of at least limited homeothermy.

Transpirational cooling is one of the most important transient avoidance mechanisms in plants (Burke and Upchurch 1989). The importance of transpiration and homeostatic control in meristem temperature has been shown for cucumber and tomato plants (Savvides et al. 2013), as well as cotton (Burke and Upchurch 1989). Our study also revealed a direct positive relationship between the transpiration rate and avoidance (Fig.18), even though we could not detect a significant effect in the combined MANOVA analysis (Table 6). Measures of transpiration rate are noisy, especially at high temperature. *Arabidopsis* populations exhibit a high level of genetic homogeneity. Most of the measured trait variance in studies of natural populations of *Arabidopsis* is between populations and regions (e.g. Montesinos et al. 2009). The certainty of assignment to population and elevation, the high trait variance among populations and regions, and the high error variance in transpiration measures mean that most of the causal variance is absorbed at the population and region level in the MANOVA, leaving little variance directly attributed to transpiration rate.

It is also important to emphasize that our transpiration rate measures were conducted on a single leaf. Transpirational cooling of the rosette involves interactions of the complex stacked leaf structure of the rosette and its interaction with the micro-climate in which plants reside. The rosette temperature depends in a complex way on the aggregate functioning of all the individual

leaves in the rosette. Whole rosette transpiration is influenced not only by the leaf properties under uniform conditions, but also on the much more complex influence of rosette structure. The structure leads to variation in the rates of energy loading to individual leaves. It also determines convective and conductive transfer of heat to the surrounding air. These factors further influence the steepness of the water vapor diffusion gradient, and temperature gradient around individual leaves. A full understanding of control over air and tissue temperature within rosettes will require study of the rosette as a functional unit.

Leaf angle did not show the significant hyponastic response we expected in this study. This is likely because we measured this trait too soon after heat stress. A constitutively steep leaf angle is a long-term adaptive trait to deal with water deficit, high radiation load, or high temperature (Fu 1989, Falster and Westoby 2003, Valiente-Banuet et al. 2010). Plants from low latitude showed much steeper leaf angle compared with high latitude plants in 21 European *Arabidopsis thaliana* ecotypes, and all plants displayed steeper leaf angle in response to extended vernalization period (10, 20, or 30 days) (Hopkins et al. 2008). Physiologically-driven changes in leaf angle can be elicited by a variety of environmental conditions, including heat. Leaf angle movement generally requires observation over a number of hours (Ehleringer 1987). However, in our study, we measured leaf angle 15 min after heat treatment started. We hypothesize the leaf angle might change if given repeated and prolonged heat stress period and measured later in the treatment.

Hsp101 and SA accumulation both had very high variation (Fig.19) in this study. In prior published studies plants were assayed for Hsp101 and SA accumulation in seedling stage when plants have not yet developed any functional avoidance mechanisms. Seedlings therefore experience a heat stress temperature that is equal to the ambient heat treatment temperature. Thus

in these prior studies, Hsp101 and SA accumulation is less variable and is distinguishable among populations (Tonsor et al. 2008, Zhang et al. 2014). However, adult plants can adopt both avoidance and tolerance in heat stress response. As shown in Fig.2, the actual rosette temperature for a 45^{o}C heat stress is 36.4^{o}C for high elevation populations vs. 38.2^{o}C for low elevation populations, on average. Hsp101 accumulation increases rapidly in the range of 34-40^{o}C among *Arabidopsis* plants collected from natural populations (Tonsor et al. 2008). The observed variation in this study in the actual rosette temperature might explain why we did not detect a significant difference in Hsp101 accumulation between low vs. high elevation populations at the 45^{o}C heat stress (Fig.19a); based on our past studies the various genetic lines used here are very likely to be variable in expression in uniform temperature, and differ in their DeltaT. As a result they are highly variable in their responses in an experiment like the one reported here. To the best of our knowledge this is the first time that Hsp101 has been quantified in adult plants that experienced variable plant tissue temperature despite uniform ambient temperature. In addition our Hsp101 measurement method and Hsp101 expression itself are both highly variable at the level of both biological and technical replicates (data not shown). In this study, we estimated the necessary sample sizes based on prior seedling experiments, under conditions in which variation in heat avoidance was not possible. However, in retrospect based on the results of this study, an estimated sample size of 96 (48 samples for each elevation group) would be needed to distinguish the difference in low and high elevation populations in their response to the 45^{o}C heat.

In a previous experiment, we detected a cline in SA in genetic lines collected along our study system's elevation gradient, when measured in a 22^{o}C environment (Zhang et al. 2015b). In the present study, even with the large variation in rosette temperature observed, we still

detected a significant difference in free SA accumulation comparing low and high elevation populations at both control and 45^{o}C heat stress temperatures (Fig.19b). The total SA accumulation for the low elevation populations was indistinguishable in the control vs. the 45^{o}C heat treatment, while the total SA value was significantly increased by about 180% in the 45^{o}C heat treatment for the high elevation populations, compared with their control (data not shown).

Hsp101 and SA expression are both rapidly up-regulated with heat stress. We observed a negative association between both Hsp101 and SA and photosynthetic rate. This suggests that the accumulation of Hsp101 and SA are up-regulated when cellular or subcellular damage is sensed by the plant, i.e. the same conditions in which photosynthesis declines.

The high avoidance ability under high temperature in adult plants, as we saw in Fig.15, indicates the necessity of connecting lab studies with more accurate reflections of field conditions. Previous heat stress response studies focus on seedlings under controlled lab conditions, yet it is at the reproductive stage that Arabidopsis and other spring annuals and biennials most often encounter high temperatures.

Because our study and others (Helliker and Richter 2008, Broitman et al. 2009, Helmuth et al. 2010) demonstrate that adult plants can maintain leaf and rosette temperature that differs substantially from ambient temperature due to the avoidance mechanisms, studies of heat tolerance and stress responses will be most fruitful if done in reference to plant tissue temperature instead of ambient temperature. However, even with these refined and comprehensive measures, mysteries still exist regarding how whole plants respond to heat stress in nature. Transcriptome sequencing data from these low vs. high elevation populations under heat stress could provide more detailed information about the gene networks for universal and regional-specific stress response.

Table 6. ANOVA table for avoidance using Rosette temperature as a dependent variable (here DeltaT was used, which is the difference between ambient temperature and rosette temperature for normality), and rosette angle, transpiration rate, as well as heat treatment and climate of origin and their interaction as potential causal factors. This model explains 94% variation we saw in avoidance.

Source	DF	Type III SS	Mean Square	F Value	Pr>F
Full Model (R^2=0.94)	32	3713.83*	116.06	108.77	<.0001
Heat treatment	1	1984.17	1984.17	1859.58	<.0001
Rosette Angle	1	8.52	8.52	7.98	0.005
Transpiration Rate	1	0.12	0.12	0.11	0.74
Elevation group	1	0.31	0.31	0.29	0.59
Population (Elevation group)	6	236.60	39.43	36.96	<0.0001
Genotype(Population * Elevation group)	21	242.67	11.56	10.83	<0.0001
Elevation group * Heat treatment	1	48.69	48.69	45.63	<0.0001
Error	240	256.08*	1.07		
Corrected Total	272	1075.98*			

SS: Sum of Squares.

Table 7. ANOVA table for tolerance using photosynthesis rate as a dependent, and Hsp101, free and total salicylic acid as well as heat treatment and climate of origin and their interaction as potential causal factors. This model explains 93% variation we saw in tolerance.

Source	DF	Type III SS	Mean Square	F Value	Pr>F
Full Model (R^2=0.93)	32	1384.38*	43.26	47.36	<0.0001
Heat treatment	1	1.95	1.95	2.13	0.14
Elevation group	1	64.53	64.53	70.63	<0.0001
Population (Elevation group)	6	243.19	40.53	44.37	<0.0001
Genotype(Population * Elevation group)	20	617.81	30.89	33.81	<0.0001
Hsp101 accumulation (log)	1	7.41	7.41	8.11	0.005
Total salicylic acid accumulation (log)	1	73.11	73.11	80.02	<0.0001
Free salicylic acid accumulation (log)	1	7.51	7.51	8.22	0.005
Elevation group * Heat treatment	1	5.90	5.90	6.46	0.01
Error	112	102.32*	0.91		
Corrected Total	144	1486.70*			

* Sum of Squares.

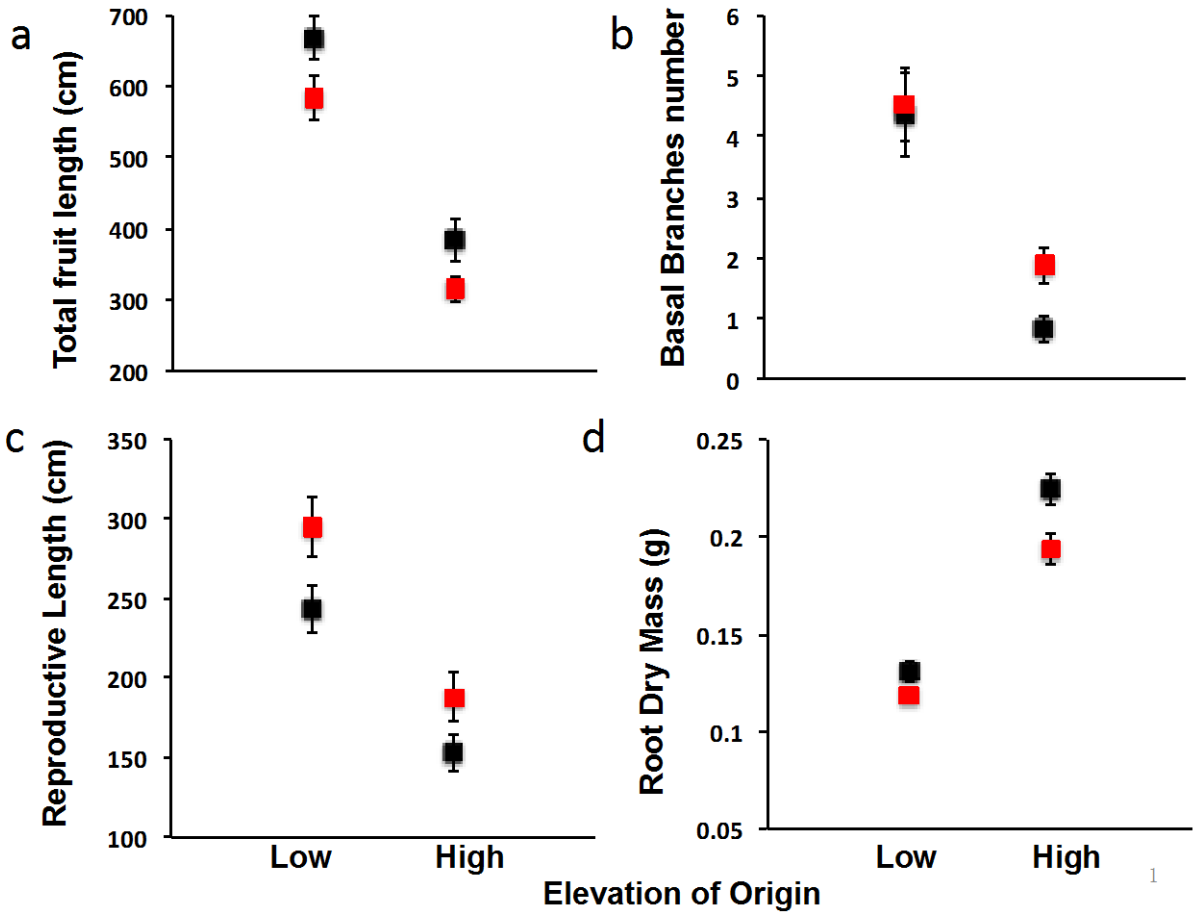


Figure 14. Heat stress disruption as measured by comparing trait values between 45ºC heat stress and control for plants originating both low elevation and high elevations. Black = control; Red = 45ºC heat stress. Figure shows the means of each elevation group under each treatment, and the bars are standard errors. See supplementary Table 1 for results from statistical analyses.

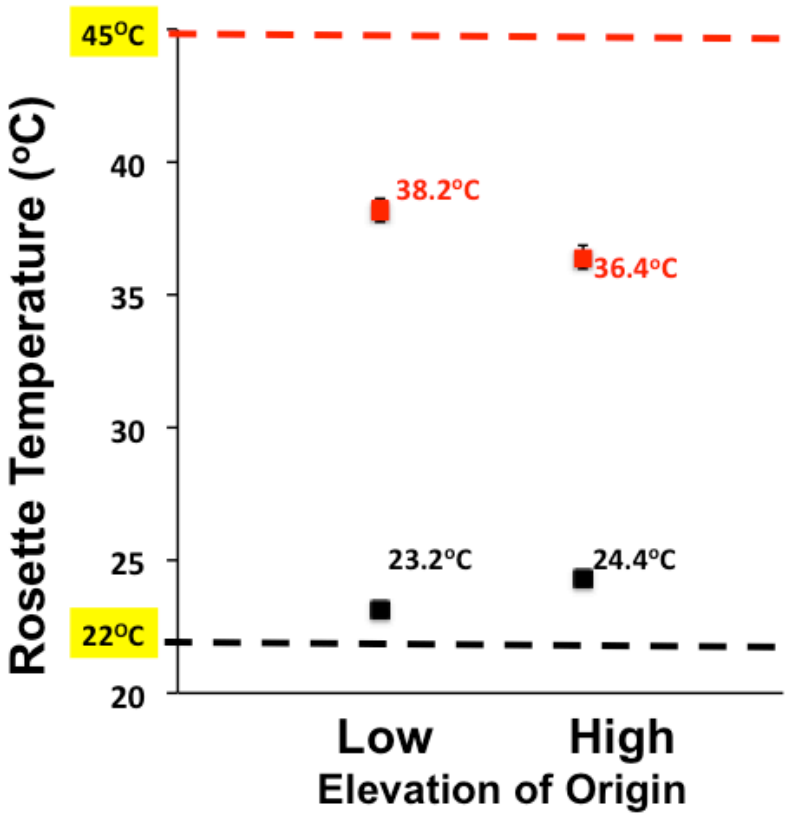


Figure 15. Rosette temperature, a measure of heat avoidance, comparing plants from low vs. high elevation populations, under 45ºC heat stress and control. Black = control; Red = 45ºC heat stress. Figure shows the means of each elevation group under each treatment, and the bars are standard errors. Dash lines show the ambient temperature for control (22ºC) and heat treatment (45ºC). Notice the standard error for the low vs. high elevation populations under the control is very small, so it is invisible in the figure.

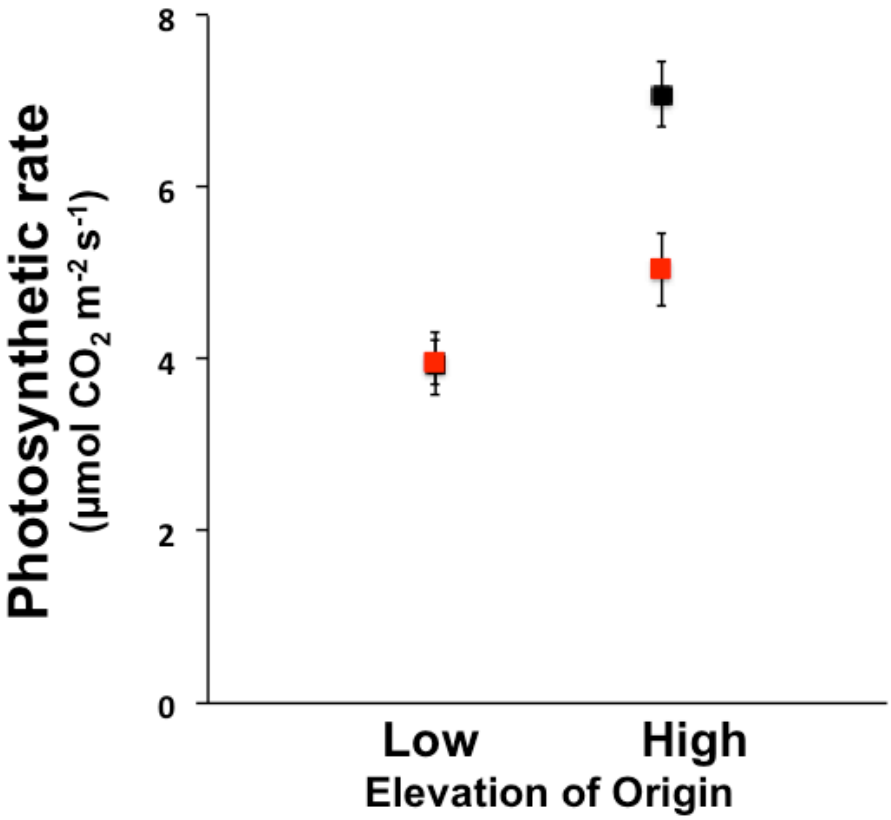


Figure 16. Photosynthetic rate, a measure of heat tolerance, for the low vs. high elevation populations under the 45ºC heat stress and control. Black = control; Red = 45ºC heat stress. Figure shows the means of each elevation group under each treatment, and the bars are standard errors. Note the photosynthetic rates for low elevation control and 45ºC heat stress treatments overlap each other.

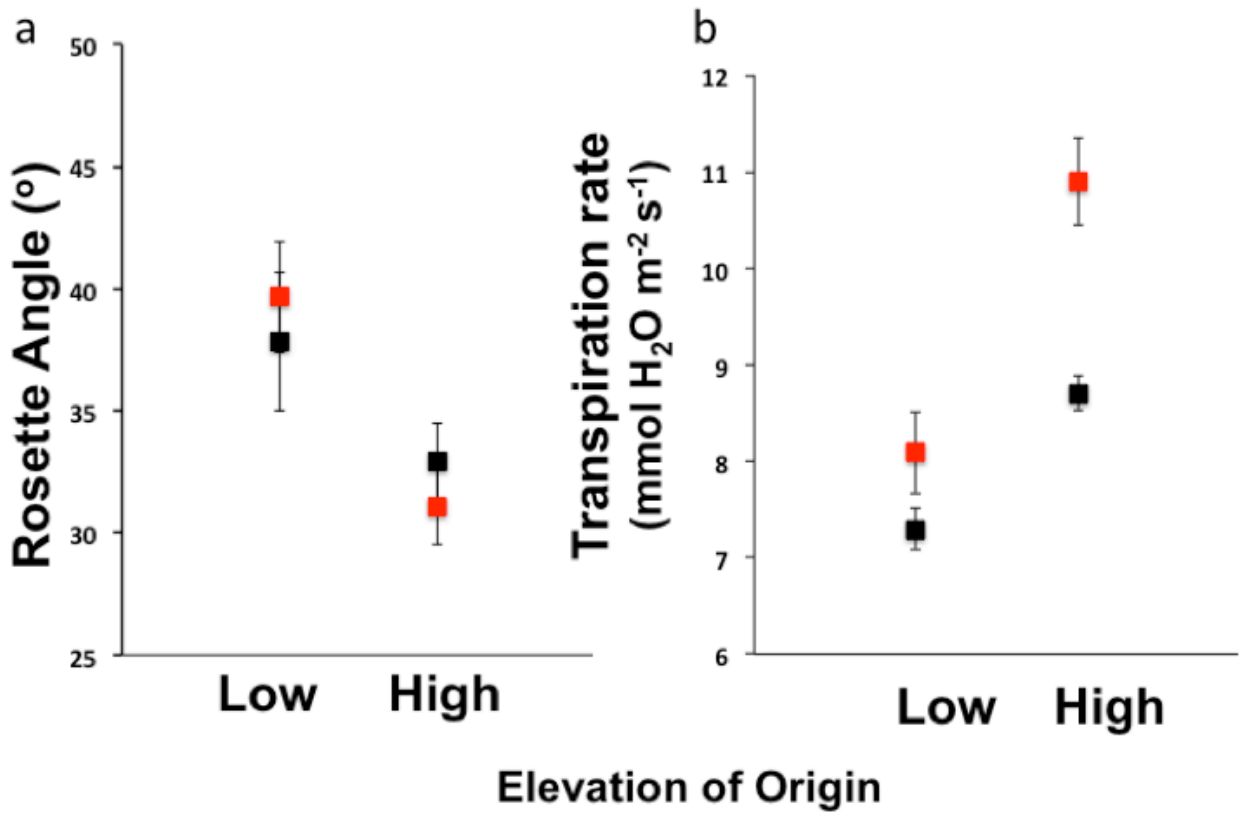


Figure 17. Two potential avoidance mechanisms compared for the low vs. high elevation populations under 45ºC heat stress and control. Black = control; Red = 45ºC heat stress. Figure shows the means of each elevation group under each treatment, and the bars are standard errors.

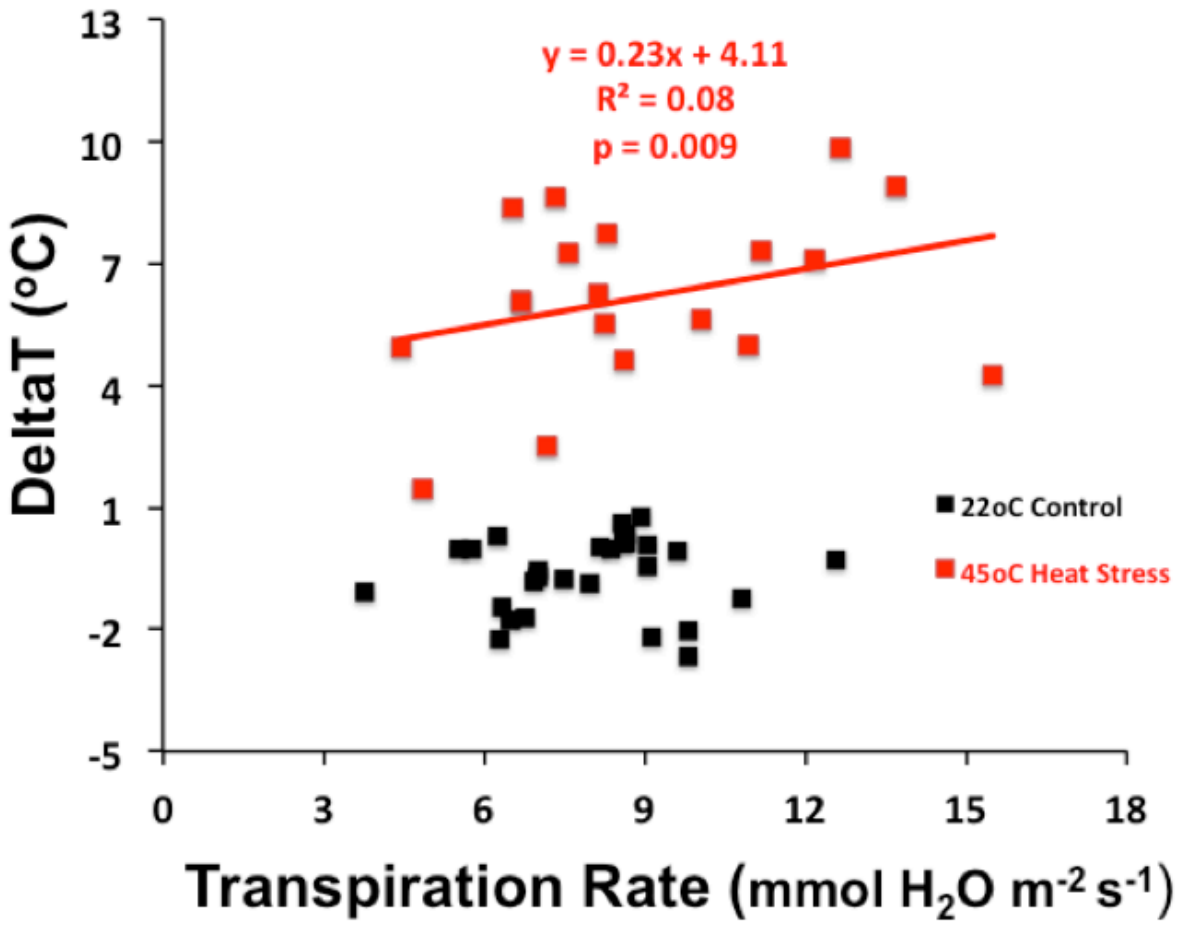


Figure 18. The relationship between transpiration rate and DeltaT (the difference between air and rosette temperature), comparing 45ºC heat stress and control. Data points displayed are genotype means at both control and heat treatment groups. Statistical analysis was done with individual plant trait values (total samples = 84) using Proc REG in SAS. The regression line for the 45ºC heat stress is: DeltaT = 0.23*Transpiration rate + 4.11. The slope is statistically significant (p=0.009).

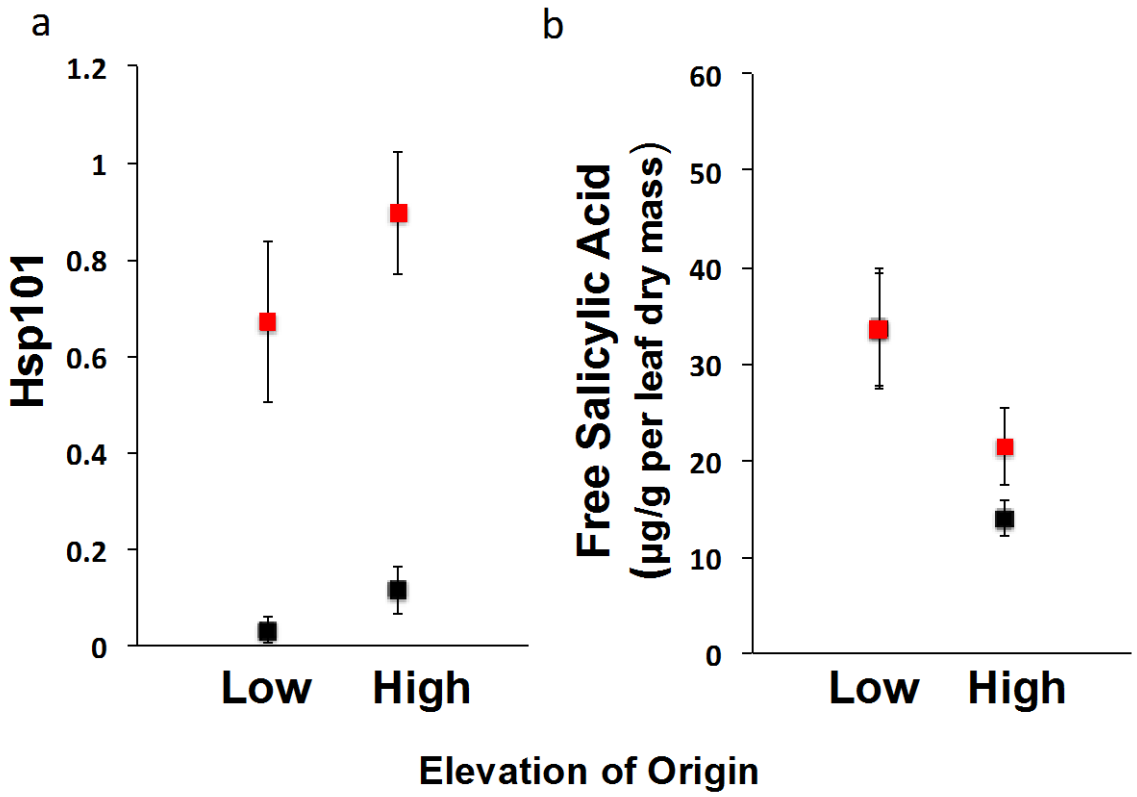


Figure 19. Two tolerance mechanisms, Hsp101 ratio and free salicylic acid, and their relative value in low vs. high elevation populations under 45ºC heat stress and control. Black = control; Red = 45ºC heat stress. Figure shows the means of each elevation group under each treatment, and the bars are standard error. Notice in b) the free salicylic acid concentration for the control overlapped with its concentration at the 45ºC heat stress. The Hsp101 ratio is its concentration relative to our biological standard, in which we heat treated a combination of leaf samples at 45ºC and used it as a quality control on gel-to-gel variation in western blot, see details on materials and methods in Tonsor et al. 2008. The unit for b) free salicylic acid in the figure is ug/g per leaf dry mass.

6.0 ADAPTIVE DIVERGENCE IN TRANSCRIPTOME RESPONSE TO HEAT AND ACCLIMATION IN *ARABIDOPSIS THALIANA* PLANTS FROM CONTRASTING CLIMATES

6.1 INTRODUCTION

Abiotic stresses are major driving forces in evolutionary diversification (Hoffmann and Parsons 1991, Hoffmann and Hercus 2000, Badyaev 2005). Diversification in adaptation to environments with contrasting patterns of stresses is important in shaping ecological structure in nature (Keller and Seehausen 2012).

Plants have evolved various abiotic stress response mechanisms at morphological, physiological and biochemical levels, with diversity in responses evidenced both within and between species (Berry and Bjorkman 1980, Yeh et al. 2012, Zhang et al. 2015a). Local adaptation to stressful environments has been extensively explored, such as adaptation to drought stress (Zhu 2002, McKay et al. 2003, Shinozaki and Yamaguchi-Shinozaki 2007, Bowman et al. 2013), salt stress (Zhu 2002, Zhao et al. 2012), cold stress (Sakai and Larcher 1987, Shinozaki et al. 2003, Beales 2004), and heat stress (Rizhsky et al. 2002, Rizhsky et al. 2004b, Kotak et al. 2007). However, few studies have explored the transcriptional variation underlying variation in phenotypic stress responses. Identification of changes in gene expression involved in diversification of abiotic stress responses is an important step in understanding the evolutionary

response to stress-mediated natural selection. Furthermore, understanding evolved variation in gene expression response to stresses at a population level can provide insight on the cause of the capacity/limit of an organisms' ability to adapt to local climate and the mechanisms of differential adaptation. In this study, we are particularly interested in the adaptive response of heat stress due to its increasing importance in global climate change events.

Heat stress response involves large scale gene reprograming at the level of the transcriptome in the context of complex regulatory networks (Dittami et al. 2009, Liu et al. 2013a). The multiple genes discovered by RNA-seq analysis among animal and plant species have suggested a complicated structure to the response to heat stress (Kotak et al. 2007). Two main pathways are activated during exposure to heat (Kotak et al. 2007, Ahuja et al. 2010, Qu et al. 2013). Heat stress first activates the up-regulation of heat shock transcription factors (Hsfs) and heat shock proteins (Hsps) (Baniwal et al. 2004, Wang et al. 2004). The highly conserved Hsps are the most extensively studied heat stress related genes. Oxidative stress, as a secondary stress, is also activated during heat stress (Qu et al. 2013). Reactive oxygen species (ROS) pathway, the expression of transcription factors in Zat and WRKY family, MBF1c and Rboh, is thus activated (Rizhsky et al. 2004a, Suzuki and Mittler 2006, Ciftci-Yilmaz et al. 2007, Suzuki et al. 2008). Several critical biological processes, such as antioxidant system neutralization of free radicals, protein synthesis and degradation, plant hormone production, are involved in the response (e.g. salicylic acid) (Liu et al. 2013a, Liu et al. 2013b, Qu et al. 2013, Narum and Campbell 2015).

The adaptive responses to climate variables are highly dependent on the geographic origin of the populations and their genetic background (Schimper 1902). Gene expression response to the application of the plant hormone salicylate varied in *Arabidopsis thaliana*

populations from diverse climate origins (Leeuwen et al. 2007). Our study region, Iberian Peninsula in NE Spain, provides an ideal location for studying the general patterns of response to climate in plants. Populations collected across an elevation gradient provide a platform to examine adaptation to diverse climates (Schimper 1902, Clausen and Hiesey 1958). Two major climate variables, annual temperature and precipitation, follow closely with elevation along a gradient from the Mediterranean coast into the Pyrennee mountains (Wolfe and Tonsor 2014). Importantly, native *Arabidopsis thaliana* populations in this region show morphological and physiological divergence, such as life cycle timing (Wolfe and Tonsor 2014), seed dormancy and germination traits (Montesinos-Navarro et al. 2012) and one key plant hormone, salicylic acid (Zhang et al. 2014). These native *Arabidopsis* populations also show divergent response to various abiotic and biotic stresses. For example, sixteen populations showed differential Hsp101 expression when exposed to a 42^{o}C compared to a 45^{o}C heat treatment (Zhang et al. 2015a), while salicylic acid differed among four tested populations when exposed to a 44^{o}C heat treatment for 3hrs(Zhang et al. 2015b). These populations also show differential expression when exposed to cold stress and pathogen infection (Zhang et al. 2014). Recently, adult plants under heat stress showed contrasting avoidance and tolerance strategies when comparing plants from contrasting climates (Zhang et al. under review). These diverse and contrasting strategies for low vs. high elevation plants suggest that adaptive and fine-tuned heat stress mechanisms are involved.

Acclimation, a process resulting from a pre-exposure to sub-lethal high temperature before exposing plants to the extreme high temperature, is an important adaptive mechanism of plant survival when in a high temperature environment (Badger et al. 1982, Alscher and Cumming 1990, Whitehead 2012). A previous microarray experiment from Larkindale and

Vierling (2008) showed that two heat treatment regimes, one with a moderately high temperature acclimation followed by high temperature, the other a direct exposure to high temperature, have very different core genome responses (Larkindale and Vierling 2008). Both the number and abundance of transcripts up-regulated and down-regulated under heat stress (compared to the control condition) differ between the two heat treatment regimes. In addition, among the multiple genes that are involved in acclimation to high temperature, there appears to be more than one strategy that achieves similar protective effects (Larkindale and Vierling 2008). Thus in our study, we looked the transcriptome response to heat stress with or without an acclimation treatment. Identifying the specific gene set in each heat stress regime and elucidating the complete mechanisms of heat stress response will contribute to fine-scale control for future breeding programs as well as for predicting the response to future climate change.

RNA-Seq has become a powerful and revolutionary tool to investigate the divergent responses to various thermal climates within species when they are exposed to the same heat stress (Wang et al. 2009). In this study, our goals were to identify whether/how plants from contrasting climates showed different gene expression patterns in response to heat and whether/how an acclimation treatment altered the gene expression patterns. To do this, we exposed low and high elevation *Arabidopsis thaliana* plants to two 45ºC heat treatments: one without and one with a 38ºC acclimation. We firstly compared the constitutive gene expression level between the low and high elevation plants in the control. We then identified elevation specific significantly differentially expressed (DE) genes by contrasting low and high elevation plants within each treatment (within heat treatment, across elevation groups). We identified the elevation specific DE genes for both treatments. Next we identified acclimation specific DE genes by comparing the two heat treatments for each elevation group (i.e. within elevation group,

across heat treatment). We specifically looked into the gene expression level of currently known heat stress related DE genes, including heat shock proteins (Hsps), heat shock transcription factors (Hsfs) and many others, in our elevation specific and acclimation specific DE genes. We also investigated the functions of the genes were DE for both low and high elevation plants but with opposite directions of changes for the plants from the two climate regions. We did this for each heat treatment. Our study shed light on evolutionary adaptation to local climates, especially past high temperature events, at the transcriptome level.

6.2 MATERIALS AND METHODS

6.2.1 *Arabidopsis thaliana* materials and treatments

Plants from eight populations, four from low and four from high elevation, were chosen as representative plants. Since *Arabidopsis thaliana* is highly selfing and highly genetically homogenous within populations (Tang et al. 2007), we selected four plants, one genotype per population, to represent the plants in each elevation region. To test for differential responses to heat and the role of acclimation, we designed two heat treatments that we compared to a control group. 24 plants total, consisting of three replicates of each of the eight unique plants, were blindly divided into the three treatment groups. All plants were germinated following a five-day 4°C stratification in the dark and maintained at 22°C for three weeks (16 hrs light/8 hrs dark) in Conviron PGW36 controlled environment growth chamber (http://www.conviron.com) at the University of Pittsburgh. After three weeks of growth, seedlings then experienced a four-week vernalization treatment at 5°C to synchronize flowering time.

Since these plants are most likely to experience heat stress at the bolting stage in nature, heat treatments were imposed at standard stage 6.0-6.1 (Boyes et al. 2001). Following vernalization, plants were observed daily and those at the stage 6.0-6.1 were selected for heat treatment in a separate PGW36 chamber. The heat treatments were: a) 45 ^{o}C: a 45^{o}C treatment for 3hrs; and b) 38/45 ^{o}C: a 38^{o}C acclimation for 3hrs followed 2hrs later with a 3hr 45^{o}C treatment. The control group was maintained at a constant 22°C throughout the experiment. Each treatment group included all eight plants. After placement of plants in the heat treatment chamber, the temperature increased over a 15 minute period from a starting temperature of 22°C, as in Larkindale and Vierling, 2008.

6.2.2 RNA extraction, cDNA library construction and RNA sequencing

Leaf samples for both heat treatment and control plants were collected immediately after the heat treatment, stored in liquid nitrogen, and quickly stored in -80oC freezer. After leaf samples were freeze-dried, RNA was extracted and purified using Qiagen RNeasy Plant Mini Kit (Qiagen) using the kit's instruction manual recommended protocol. The quality and quantity of the RNA samples were measured using Qubit Fluorometric quantitation (Thermo Fisher Scientific). Then total RNA samples were adjusted/diluted to 100ng/ul in 25ul nuclease free water (2.5 ug total) for cDNA library construction. Before cDNA library construction, all RNA samples were evaluated via Bioanalyzer for further RNA quality assessment (Genomics Research Core, Health Science Core Research Facilities, University of Pittsburgh). RNA samples were re-extracted and re-purified if they did not pass the quality control.

Next the poly-A RNAs were converted into ds-cDNA and fragmented into 100bp fragments. cDNAs were then ligated with adaptors and amplified with PCR. The cDNA libraries

were constructed using the Truseq RNA Sample Prep kit (Illumina) in the DNA Core, University of Missouri.

Eight cDNA libraries were combined per pool, three pools total. Each pool was sequenced in a single lane of a 1x100bp single-end Illumina HiSeq 2000 run, 3 lanes total. The sequencing was done in November 2014 at the University of Missouri DNA Core.

6.2.3 RNA sequence mapping and differential expression

The raw read data were first checked with FastQC software for quality control. Because the per base QC content was high for the first 15bp, the sequences were processed with FastX Trimmer to trim the first 15bp and last 15bp for each 100bp sequence for high quality sequence alignment.

The reads were then mapped to *Arabidopsis thaliana* Col-0 (Tair-10) transcriptome with Tophat2. Two mismatches were allowed in each segment alignment for reads mapped independently. We obtained an average of 28M raw reads per sample. An average of 23.7M reads, 85.8% of the raw reads, were mapped to the reference genome (Table 26).

To understand the constitutive gene expression between low and high elevation plants, we firstly identified the up- and down- regulated significantly differentially expressed (DE) genes by contrasting high elevation plants with low elevation plants in the control. The comparison and the functional annotation was performed using CuffDiff2 with $p = 0.01$ (Trapnell et al. 2012).

Next, to categorize the DE genes, we adopted two approaches. For both approaches, we firstly calculated the difference in gene expression between each heat treatment and the control, separately for low or high elevation plants. The difference indicates differential gene expression (DE) response to the heat treatments. Our first approach was to contrast the gene expression

response of low and high elevation plants to each heat treatment. The second approach was to compare the gene expression in 45 ºC and 38/45 ºC to investigate the role of 38 ºC acclimation. We contrasted the gene expression of 45 ºC and 38/45 ºC within elevation groups, comparing for low and high elevation plants respectively. The detection of DE genes, their fold change, and normalized fold change (FPKM- Fragments Per Kilobase of Exon per Million Fragments Mapped), as well as their functional annotations, were conducted using Cuffdiff2 with p value = 0.01 (Trapnell *et al.* 2012). For all comparisons, we firstly contrasted all the DE genes regardless of the direction of change. We then compared up-regulated and down-regulated DE genes separately. By doing the above comparison, we also uncovered DE genes that exhibited opposite direction of change between elevation groups. The DE shared across elvation groups and the DE genes that were unique to an elevation group were compared and visualized using BioVenn, a web application (http://www.cmbi.ru.nl/cdd/biovenn/index.php). The functional categorization of the DE genes was performed in TAIR (https://www.arabidopsis.org/tools/bulk/go/index.jsp).

We furthered explored the elevation specific and acclimation specific DE genes from the above comparisons. We investigated the currently known stress-related genes in the Hsp/Hsf pathway and the ROS pathway from a literature review list (Table 27). We used these known heat stress related genes and their magnitude of change in heat to represent the elevation or acclimation specific response in our study. In our approach of comparing DE genes regardless of direction with separate up- and down-regulated DE gene, we also uncovered 51 shared DE genes DE between high and low elevation plants, but with directions of change (35 DE genes in 45ºC, 19 DE genes in 38/45 ºC, with three shared DE genes; Table 4). Their function and possible biological processes involved were also double checked in NCBI database (http://www.ncbi.nlm.nih.gov).

6.3 RESULTS

6.3.1 Constitutive gene expression difference in low vs. high elevation plants

When expression levels in high elevation plants were compared to low elevation plants in the control, 1291 DE genes were found. Of these, 826 were up-regulated and 465 were down-regulated in the high elevation plants relative to low elevation plants. We found eight Hsps, including Hsp60, Hsp70 and Hsp90 family, that showed up-regulation in high elevation plants (Table 8). Zat7 and Zat10, responding to various stresses, were also up-regulated in high elevation plants. ABI2, involved in abscisic acid (ABA) signaling, NDH1, providing protection against photo-oxidation, and FtsH11, associated with reduced photosynthetic capacity in heat stress, were down-regulated in high elevation plants. Hsps respond via Hsp/Hsf pathway, Zat genes respond to ROS pathways. The up-regulation in Hsps and Zat indicates that high elevation plants were constitutively more resistant to heat stress. Down-regulation in NDH1 and FtsH11 indicates a negative control in photosynthesis in high elevation plants.

In plants, MADS-box genes play major roles in controlling development and determining flowering time. The MADS-box gene FLOWERING LOCUS C (FLC) and SOC1 (AGL20, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1) are necessary for the correct flowering timing. In high elevation relative to low elevation plants, FLC gene was up-regulated and the SOC1 was down-regulated. Another MADS-box gene AGL3 was also down-regulated. The constitutive gene expression difference in MADS box genes indicates inherent variation in flowering time between low and high elevation plants.

6.3.2 Elevation-specific DE genes in response to heat

When exposed directly to a 45 oC heat, 1516 and 2104 DE genes were found for low and high elevation plants, respectively (Fig.20a). Similarly, when exposed to the 38/45 oC heat, 1489 and 1972 DE genes were found for low and for high elevation plants, respectively (Fig.20b). High elevation plants showed 39% and 32% more DE genes than low elevation plants in 45 oC and 38/45 oC heat, respectively. High elevation plants in the 45 oC heat showed the most DE genes. We further compared the magnitude of fold change in low and high elevation plants in the two heat treatments (Fig.21). High elevation plants in the 45 oC heat also showed the largest average magnitude of fold change among the four bars. The average magnitude of fold change was similarly low.

When the DE genes in the 45 oC heat were compared between the two elevation groups, 494 shared DE genes were identified. Among these 494 shared DE genes, 161 were up-regulated, 298 were down-regulated, and 35 showed directions of change. For the elevation specific DE genes, high elevation plants had 58% more uniquely DE genes than low elevation plants (1610 vs. 1022, Fig.20a). Similarly, we found 602 shared DE genes between low vs. high elevation plants after the 38/45oC heat. 284 of these were up-regulated, 299 were down-regulated, and 19 showed opposite directions of change in the two elevation groups. High elevation plants have 54% more unique DE genes than low elevation plants (1370 vs. 887) (Fig.20b). We further compared the numbers of up-regulated and down-regulated DE genes between the two elevation groups. In 45 oC, both low and high elevation plants showed more elevation-specific up-regulated (573 and 850) than down-regulated DE genes (484 and 795). However, in 38/45 oC, there were more high elevation specific down-regulated DE genes (1088)

than up-regulated DE genes (301), while more low elevation-specific up-regulated (610) than down-regulated DE genes (296) (Fig.20).

To further explore the elevation-specific DE genes, we investigated the expression level of each gene in Table 26 from the above-mentioned elevation-specific DE genes (Fig.20), for each heat treatment (Table 9). Genes in Table 26 include genes involved in Hsp/Hsf pathway and ROS pathway. In 45 oC, we found 10 low elevation-specific and 22 high elevation-specific DE heat stress related genes. In low elevation plants, several small Hsps, such as Hsp20, Hsp21, Hsp22, had the largest up-regulation; while in high elevation plants, the Hsp70 and Hsp 90 sub-families had the largest up-regulation. Low elevation plants also differentially expressed Hsp60, Hsp70, and Hsp90 sub-family genes, but with different DE genes within the sub-families compared to high elevation plants and with significantly lower magnitude of fold change. Hsp101, the only Hsp known to be necessary for acquired thermotolerance (Hong and Vierling 2001), was uniquely expressed in low elevation plants only. Only one Hsf showed down-regulation in low elevation plants but five Hsfs showed up-regulation in high elevation plants, High elevation plants also showed DE in eight ROS related genes. DREB2 and DREB2B were up-regulated. Up-regulation of DREB genes activates the expression of stress-related genes. RBohD and RBohF were ROS signal amplifiers and were down-regulated. DGD1 and DGD2, whose expression were associated with reduction in photosynthetic capacity, were up-regulated. To summarize, in low elevation plants, only the Hsp/Hsf pathway was activated and small Hsps had the highest magnitude of change; in high elevation plants, both Hsp/Hsf and ROS pathways were activated, with Hsp70 and Hsp90 showing the largest magnitude of fold change.

In 38/45 oC, we found ten low elevation-specific and five high elevation-specific DE heat stress related genes. Low elevation plants showed up-regulation in small Hsps, Hsp60s and

Hsp70s, and small Hsps showed much higher fold change than Hsp60s and Hsp70s. High elevation plants showed down-regulation in three Hsps (Hsp70, Hsp81-3 and Hsp15.4). Low elevation plants also showed up-regulation in DREB2 and BOB1 gene, increasing stress related genes. However, NDH-M gene was down-regulated in low elevation plants, potentially affecting photo-oxidation protection. In high elevation plants, Zat7 showed down-regulation. This might reduce the amount of antioxidant produced via the ROS pathway. High elevation plants also showed up-regulation in ABA signaling factor ABI2. In summary, in 38/45 oC, low and high elevation plants were activated in both the Hsp/Hsf and the ROS pathway. Low elevation plants had up-regulation in all Hsps, especially small Hsps; high elevation plants had down-regulation in the Hsps. Low elevation and high elevation also adopted different genes in the ROS pathway.

6.3.3 Acclimation-specific DE genes in low and high elevation plants

To uncover acclimation-specific DE genes in low and high elevation plants separately, we contrasted DE genes in 45 oC with 38/45 oC for plants from each elevation. The DE genes that were uniquely expressed in 38/45 oC for plants from each elevation were acclimation-specific genes. We found 953 shared DE genes between the two heat treatments and 536 acclimation-specific DE genes for low elevation plants (Fig. 22a). Among the 536 acclimation-specific DE genes, 303 were up-regulated and 233 were down-regulated. We found 947 shared DE genes and 1025 acclimation-specific DE genes in high elevation plants (Fig. 22b). Among the 1025 acclimation-specific DE genes, 341 were up-regulated and 695 were down-regulated. High elevation plants showed more acclimation-specific DE genes than low elevation plants.

To further explore the acclimation-specific DE genes, we investigated the expression level of each gene in Table 26 from the above-mentioned acclimation-specific DE genes

(Fig.21), for plants from each elevation (Table 10). Genes in Table 26 include genes involved in Hsp/Hsf pathway and ROS pathway. There were seven and eight acclimation-specific DE genes for low and high elevation plants respectively (Table 10). In the seven acclimation-specific DE genes in low elevation plants, only two Hsps, Hsp70 and BIP3, and one Hsf, HspA1e, were up-regulated. Two DREB genes also showed up-regulation. In the eight acclimation-specific DE genes in high elevation plants, six Hsps were up-regulated, with small Hsps having the largest magnitude of fold change. High elevation plants also experienced mostly down-regulation in the ROS pathway, such as Zat7. Hsps in high elevation plants also showed much higher magnitude of change than low elevation plants. The difference in expressed Hsps and other genes in ROS pathway showed that with acclimation, low elevation plants mainly adopted up-regulation in Hsp70s in Hsp/Hsf pathway and DREB genes in ROS pathway; high elevation plants adopted up-regulation in small Hsps, Hsp60s, Hsp70s, and Hsp101 in Hsp/Hsf pathway and ABA signaling in ROS pathway.

6.3.4 DE in both high and low elevation plants, but opposite directions of change

There were shared DE genes between low and high elevation plants that show opposite directions of change. There were 35 genes that were differentially expressed in the 45 oC treatment (Fig.20a) and 19 in the 38/45 oC treatment (Fig.20b) for which expression change was in opposite directions in the two elevation groups (Table 11). The functions of these genes mainly involve response to abiotic or biotic stress (such as heat, cold, chitin, ethylene stimulus, and wounding), signal transduction, biosynthetic processes, oxidation-reduction processes and cell redox homeostasis.

Of the 35 and 19 DE genes showing opposite directions of expression in the two elevation groups, three genes were included among both the 35 and 19 genes of this type from the two heat treatments: AT5G45340, AT5G54380, AT3G57450. Their functions primarily relate to abscisic acid (ABA)-activated signaling pathways, associated with response to abiotic or biotic stress. In the 35 shared DE genes at 45 oC, nine DE genes showed up-regulation in the low elevation plants but down-regulation in the high elevation plants. The remaining 26 out of 35 DE genes showed down-regulation in the low elevation plants but up-regulation in the high elevation plants. However, all 19 shared DE genes at 38/45 oC heat showed down-regulation in the low elevation plants but up-regulation in the high elevation plants. Among the 51 genes (that is: 35 + 19 – 3 overlapping), about 30 have been categorized as response to abiotic or biotic stresses (Table 11). These shared DE genes with opposite directions of change further showed the diversification in response to heat stress between low and high elevation populations.

6.4 DISCUSSION

When plants are exposed in high temperature, they not only experience heat stress, a secondary stress, oxidative stress, is also activated. Thus both genes in Hsp/Hsf pathway and in reactive oxygen species (ROS) pathway, including antioxidant and plant hormones, are produced (Qu et al. 2013). Here we compared the gene expression patterns for low and high elevation plants in NE Spain, in response to 45^{o}C and 38/45 ^{o}C treatments. High elevation plants had constitutively higher heat stress gene expression level, in both Hsp/Hsf and ROS pathway. In 45^{o}C, only the Hsp/Hsf pathway was activated and small Hsps had the highest magnitude of change in low elevation plants; both Hsp/Hsf and ROS pathway were activated, with Hsp70 and Hsp90 showed

the largest magnitude of fold change, in high elevation plants. in 38/45 ^{o}C, low and high elevation plants were activated in both Hsp/Hsf and ROS pathway. Low elevation plants had up-regulation in all Hsps, especially small Hsps; high elevation plants had down-regulation in the Hsps. Low elevation and high elevation also adopted different genes in the ROS pathway. We also discussed shared genes between low and high elevation plants but with directions of change. This study indicates that low and high elevation plants have evolved adaptive divergence in heat stress response. The contrasting patterns of temperature variation in low and high elevation sites appears to have played a strong role in the evolution of divergent patterns of both pre-acclimation and direct exposure gene expression responses to high temperature stress.

6.4.1 Population divergence in response to heat stress

Even when populations were not under heat stress, there was significant divergence in gene expression. This could potentially explain much of the phenotypic variation, such as flowering time, seed size, we documented previously in plants from the present study populations and others in this region (Montesinos-Navarro et al. 2009, Montesinos-Navarro et al. 2011, Montesinos-Navarro et al. 2012, Wolfe and Tonsor 2014). For example, low elevation plants flowers early but high elevation plants take a longer time to flower (Wolfe and Tonsor 2014). This can be explained by the expression of MADS box gene FLC and SOC1. FLC is a repressor of flowering (Michaels and Amasino 1999) and SOC1 promotes flowering (Lee and Lee 2010). FLC gene was up-regulated and the SOC1 was down-regulated in high elevation plants relative to low elevation plants, thus repressing flowering.

Natural populations of redband trout from desert sites showed the most uniquely differentially expressed transcripts and most abundant differentially expressed genes compared

with populations from montane environment when exposed to severely high water temperatures (Narum and Campbell 2015). In response to a common thermal environment for intertidal snail *Chlorostoma funebralis*, more stress-responsive genes were observed in northern populations than southern populations (Gleason and Burton 2015). In the native environment of our samples, low elevation plants experience hot and dry climate while high elevation plants experience cold and wet conditions (Montesinos-Navarro et al. 2009, Montesinos-Navarro et al. 2011, Montesinos-Navarro et al. 2012, Wolfe and Tonsor 2014). Thus we hypothesize that low elevation plants potentially evolve to be more adapted to heat stress, through acclimation to more frequent high temperature events, but high elevation plants are more sensitive. Our data supports this hypothesis. High elevation plants expressed more elevation specific DE genes than low elevation plants in both heat treatments (Fig.20). In 45ºC heat, high elevation plants showed more currently known heat stress related elevation specific DE genes than low elevation plants. However, with acclimation, low elevation plants showed up-regulation in Hsps but high elevation plants showed down-regulation in Hsps (Table 9). It is also worthwhile to notice that for high elevation plants in 38/45ºC heat, there were 1088 elevation specific down-regulated DE genes, indicating substantial gene down-regulation involved (Fig. 20b).

Our phenotypic measures on the same set of biological replicates used in this study showed contrasting avoidance and tolerance strategies in a 45ºC heat stress response. High elevation populations showed more avoidance, with lower rosette temperature at heat stress; and low elevation populations adopted more tolerance, i.e. a relatively higher photosynthetic rate (Zhang et al. under review). Avoidance mechanisms include rosette angle and transpirational cooling, and tolerance mechanisms involve heat shock proteins, and plant hormones in the ROS (reactive oxygen species) pathway (Zhang et al. under review). However, to the best of our

knowledge, in our investigation on the current known heat stress related genes, the genes listed on Table 26 were all about mechanisms involved in tolerance, we did not find genes related with avoidance, in stress response. Low elevation plants had higher tolerance in response to 45^{o}C heat, by expressing more DE genes (Fig. 20a, Table 9).

6.4.2 Role of acclimation n heat stress response

Acclimation, from previous exposure to a sub-lethal high temperature, is an important adaptive mechanism and can enhance the ability to resist heat stress. Long-term acclimation can reach a new steady state with cost-effective strategies for stress response (Logan and Somero 2010).
When we compare 38/45 oC with 45 oC heat, we can look the genes specifically involved in acclimation. In 38/45 oC, both low and high elevation plants showed fewer DE genes than the number observed in response to direct exposure to 45 oC heat (Fig.21, Table 10). When we looked at the acclimation specific DE genes, low and high elevation plants had very different acclimation specific DE genes. Low elevation plants had 536 acclimation specific DE genes and high elevation plants had 1025 acclimation specific DE genes (Fig.21). For the currently known heat stress related DE genes among the acclimation specific DE genes, low elevation plants adopted two Hsps and one Hsf, but high elevation plants adopted six Hsps. Low elevation and high elevation plants also adopted very different genes in ROS pathways.

Hsp101 was previously reported to be the only Hsp that was necessary for heat tolerance (Hong and Vierling 2001). Here, we only found Hsp101 in low elevation plants in the 45oC heat, and in the high elevation plants in the 38/45oC heat. Hsp101 showed no up-regulation from 22 oC - 34 oC, but showed significant increase in 40oC compared with 34 oC (Tonsor et al. 2008). When exposed to a 45oC heat stress, low elevation plants experienced average 38.2oC but high

elevation plants experienced average 36.4oC because of avoidance mechanisms, such as transpirational cooling and inherent leaf angle change (Zhang et al. under review). The low temperature for high elevation plants, average 36.4oC, might not be high enough to activate Hsp101 expression. The difference in Hsp101 expression also showed very different mechanisms in stress response and acclimation between low and high elevation plants.

6.4.3 Adaptive divergence of Hsps and Hsfs

We found that populations from low elevation, a hot and dry environment compared to the environment of high elevation plants (Montesinos-Navarro et al. 2009, Montesinos-Navarro et al. 2011, Wolfe and Tonsor 2014), showed lower Hsp101 expression than populations from high elevation, cold and wet environment in the 45 ^{o}C heat treatment, although the difference did not reach statistical significant because of high variation in expresion at high temperature (Zhang et al. 2015a). This is in accordance with our gene expression data here. We found that low elevation plants showed fewer DE genes for Hsps in the heat treatments, compared to high elevation plants.

A similar pattern has been seen in redband trout (Narum et al. 2013), common killifish (Fangue et al. 2006) and intertidal snail (Gleason and Burton 2015). Studies on redband trout also showed lower Hsp expression observed in desert strains compared to montane strains (Narum et al. 2013, Narum and Campbell 2015). Studies on common killifish showed significantly greater Hsp70-2 gene expression in the northern than the levels observed in southern killifish populations (Fangue et al. 2006). Hsp70 is involved in negative regulation of heat stress response (Morimoto 1998). These studies combined indicate populations from warm environments might have evolved heat tolerance mechanisms with lower costs than Hsps

production. However, studies in common killifish also showed other Hsps, such as Hsp70-1, or Hsp90, had different patterns in response to heat stress, suggesting that Hsps have complex networking patterns in heat stress responses. In response to a common thermal environment for intertidal snail *Chlorostoma funebralis*, the two regions also showed important differences in the genes that were up-regulated. Hsp70s were significantly increased in the northern populations while Hsp40s were significantly up-regulated in the southern populations (Gleason and Burton 2015). This is also in accordance with our findings on Hsps in this study, in which we saw various magnitudes of gene expression and different Hsps in low and high elevation plants (Table 9).

6.4.4 Adaptive divergence in natural populations: ROS pathway

Although Hsp/Hsf pathway is still the major differentiated pathway between low and high elevation plants in heat stress response, other genes involved in ROS pathway also played a significant role in each elevation plant. Previous heat stress studies showed DE genes involve Hsps, and genes involved in ubiquitination and proteolysis (Schoville et al. 2012), as well as genes involved in oxygen transport, protein synthesis, folding and degradation in Saccharina japonica and catfish (Liu et al. 2013a, Liu et al. 2013b). Abscisic acid, salicylic acid, hydrogen peroxide and ethylene related signaling pathways are also involved in heat stress response (Larkindale et al. 2005, Larkindale and Huang 2005).

In elevation specific DE genes and acclimation specific DE genes, we found DE genes involved in ROS pathway, such as DREB2 and ABI gene (Table 2, Table 3). The shared DE genes with directions of change in gene expression in low vs. high elevation plants showed genes involved in ubiquitination (e.g., AT1G14200, AT5G55620), abscisic acid catabolic process (e.g.,

AT5G45340) and ethylene biosynthetic process or ethylene activated signaling pathway (e.g., AT4G24570, AT4G29780) (Table 4). Heat stress response is a complex process and it will certainly need more effort to clarify the genes involved and how they interact to determine phenotypic responses.

In nature, plants often face a combination of several stresses. However, plants' response to stress combinations cannot be directly predicted from the response in each single stress (Rizhsky et al. 2002, Rizhsky et al. 2004b). The next steps of research should focus on two areas. One is to understand the cross-talk among various stress responses; the second is to understand the evolution of heat stress response and acclimation in plants from various climates. Since plants originate from different climates, they experience very different patterns of stress combination, thus they evolve differently in stress response. Looking into the agriculturally important stress combinations from the stress matrix (Mittler 2006) is the next challenge.

Table 8. Constitutive expression difference in currently known heat stress related genes comparing high to low elevation plants.

Gene ID	Gene Name	FPKM
AT5G56030	Hsp90-2	292.6
AT3G12580	Hsp70	110.86
AT3G23990	Hsp60-3B	78.1
AT3G07770	Hsp89.1	36.33
AT2G33210	Hsp60-2	23.65
AT4G21870	Hsp class V 15.4	127.13
AT4G30350	Hsp	58.65
AT5G51440	Hsp23.5	4.92
AT3G46090	Zat7	30.57
AT1G27730	Zat10	671.6
AT1G21910	DREB26	17.72
AT5G57050	ABI2	-25.22
AT1G15980	NDH1	-310.35
AT5G53170	FtsH11	-78.47

Note: FPKM, short for Fragments Per Kilobase Of Exon Per Million Fragments, is the normalized fold changes in gene expression when comparing high elevation plants to low elevation plants. Positive value means up-regulation, and negative value means down-regulation.

Table 9. Currently known heat stress related elevation-specific DE genes in two treatments.

Gene ID	Gene Name	FPKM
45 °C : Low Elevation Specific DE genes		
AT1G53540	Hsp20-like chaperone	2262.1
AT4G10250	Hsp22	1162.96
AT4G27670	Hsp21	978.89
AT1G07400	Class I Hsp	750.76
AT3G12580	Hsp70	337.72
AT5G52640	Hsp90-1	302.21
AT1G16030	Hsp70b	181.3
AT1G74310	Hsp101	68.98
AT2G33210	Hsp60-2	38.47
AT4G18880	HsfA4A	-112.96
45 °C : High Elevation Specific DE genes		
AT3G09440	Hsp70-3	9602.28
AT5G56030	Hsp90-2	5385.59
AT5G56010	Hsp81-3	2079.65
AT1G79920	Hsp70	1118.23
AT4G32208	Hsp70 family protein	390.98
AT1G79930	Hsp91	198.77
AT1G11660	Hsp70 family protein	72.7
AT1G09080	BIP3 (Hsp70 protein BiP chaperone BIP-L)	19.82
AT5G62020	HsfB2A	106.37
AT4G27890	Hsp20-like chaperone	37.07
AT4G36990	Hsf4 (HsfB-1)	760.26
AT3G51910	HsfA7A	503.76
AT3G02990	HsfA1e	49.49
AT4G17750	Hsf1	25.2
AT5G05410	DREB2	742.68
AT3G11020	DREB2B	150.12
AT5G53400	BOB1	297.75
AT5G47910	RbohD	-173.85
AT1G64060	RbohF	-15.86
AT4G37925	NDH-M	274.36
AT3G11670	DGD1	121.13

AT4G00550	DGD2	58.46
38/45 °C : Low Elevation Specific DE genes		
AT4G27670	Hsp21	2548.01
AT4G10250	Hsp22	1759.95
AT3G12580	Hsp70	804.75
AT3G23990	Hsp60-3B	155.69
AT4G32208	Hsp70 family protein	49.29
AT1G11660	Hsp70 family protein	47.62
AT1G09080	BIP3	20.25
AT5G05410	DREB2	66.37
AT5G53400	BOB1	70.0
AT4G37925	NDH-M	-243.67
38/45 °C : High Elevation Specific DE genes		
AT5G02490	Hsp70	-267.73
AT5G56010	Hsp81-3	-248.96
AT4G21870	Hsp15.4	-105.18
AT3G46090	Zat7	-27.83
AT5G57050	ABI2	26.13

Note: FPKM, short for Fragments Per Kilobase Of Exon Per Million Fragments, is the normalized fold changes in gene expression when comparing high elevation plants to low elevation plants. Positive value means up-regulation, and negative value means down-regulation.

Table 10. Acclimation Specific heat stress related DE genes for low and high elevation plants.

Gene ID	Gene Name	FKPM
Acclimation specific stress related DE genes in low elevation plants		
AT1G11660	Hsp70 family protein	47.62
AT1G09080	BIP3	20.25
AT3G02990	HsfA1e	18.38
AT5G05410	DREB2	66.37
AT3G11020	DREB2B	46.27
AT5G53400	BOB1	70
AT4G37925	NDH-M	-243.67
Acclimation specific stress related DE genes in high elevation plants		
AT1G53540	Hsp20-like protein	2411.27
AT1G07400	Class I Hsp	849.01
AT1G16030	Hsp70b	249.9
AT1G74310	Hsp101	101.04
AT2G33210	Hsp60-2	72.38
AT5G02490	Hsp70	-267.73
AT3G46090	Zat7	-27.83
AT5G57050	ABI2	26.13

Note: FPKM, short for Fragments Per Kilobase Of Exon Per Million Fragments, is the normalized fold changes in gene expression when comparing high elevation plants to low elevation plants. Positive value means up-regulation, and negative value means down-regulation.

Table 11. DE genes that were shared between low and high elevation plants but different directions.

Gene ID	Gene Name	Low	High	Gene description
45oC				
AT1G01120	KCS1	74.09	-106.05	3-ketoacyl-CoA synthase 1.Encodes a condensing enzyme KCS1 (3-ketoacyl-CoA synthase 1) which is involved in the critical fatty acid elongation process in wax biosynthesis.
AT3G20820		98.94	-34.96	leucine-rich repeat-containing protein (involve in: defense response, signal transduction)
AT3G57450		67.27	-353.44	hypothetical protein (involve in: abscisic acid-activaed signaling pathway, jasmonic acid biosynthetic process, response to chitin, fungus, jasmonic acid and wounding)
AT4G08950	EXO	18.82	-63.25	Phosphate-responsive 1 family protein (involve in:response to brassinosteroid,sterol biosynthetic process)
AT4G15200	FH3	124.48	-27.56	formin 3.Actin nucleation factor that directs the formation of actin cables in pollen tubes. Involved in cytoplasmic streaming and polarized growth in pollen tubes.
AT5G07030		14.26	-11.87	aspartyl protease family protein
AT5G45340	CYP707A3	18.93	-38.47	abscisic acid 8'-hydroxylase 3 (involve in: abscisic acid catabolic process, oxidation-reduction process, response to chitin, to red or far red light and to water deprivation)
AT5G47330		51.93	-176.76	palmitoyl protein thioesterase family protein
AT5G54380	THE1	16.57	-12.45	receptor-like protein kinase THESEUS 1 (involve in: response to brassinosteroid)
AT3G49580	LSU1	-31.89	136.79	response to low sulfur 1 protein
AT1G01830		-27.83	55.6	armadillo/beta-catenin-like repeat-containing protein
AT1G04350		-170.61	121.15	encodes a protein whose sequence is similar to 2-oxoglutarate-dependent dioxygenase
AT1G14200		-29.88	340.09	RING finger domain-containing protein (Annotated as response to heat)
AT1G15410		-35.45	185.01	aspartate-glutamate racemase-like protein
AT5G55620		-78.64	109.6	hypothetical protein (involve in: cellular response to ethylene stimulus, to iron ion, and to nitric oxide)
AT5G55970		-30.32	19.19	RING/U-box domain-containing protein
AT1G18330	EPR1	-26.82	33.14	early-phytochrome-responsivel
AT1G19960		-525.01	421.28	hypothetical protein
AT1G21000		-222.96	163.81	PLATZ transcription factor domain-containing protein
AT1G26800		-26.09	292.38	RING/U-box domain-containing protein (function in zinc ion binding)
AT1G29700		-104.67	126.95	metallo-beta-lactamase domain-containing protein
AT1G48300		-449.54	266.3	hypothetical protein(involve in: triglyceride biosynthetic process.Triglyceride,TG or TAG,is an ester derived from glycerol and three fatty acids)
AT3G04060	NAC046	-22.09	31.29	NAC domain containing protein 46 (involve in heat acclimation)
AT3G08860	PYD4	-11.51	40.91	PYRIMIDINE 4(Encodes a protein that is predicted to have beta-alanine aminotransferase activity).
AT3G10420	SPD1	-132.95	112.24	Protein seedling plastid development 1
AT3G16150		-37.57	45.83	probable Iprobable isoaspartyl peptidase/L-asparaginase 2
AT3G18950		-26.37	41.53	WD40 domain-containing protein
AT3G62960		-112.95	83.56	glutaredoxin-C14 (involve in cell redox homeostasis, oxidation-reduction process)
AT4G13830	J20	-296.55	240.44	chaperone protein dnaJ 20
AT4G19170	NCED4	-468.9	185.84	nine-cis-epoxycarotenoid dioxygenase 4 (involve in:anthocyanin-containing compound biosynthetic process, and oxidation-reduction process).

AT4G20070	AAH	-46.04	49.1	allantoate deiminase
AT4G33040		-90.15	77.17	glutaredoxin-C6 (involve in cell redox homeostasis, oxidation-reduction process)
AT5G01820	SR1	-113.74	111.56	CBL-interacting serine/threonine-protein kinase 14
AT5G23750		-56.02	47.43	Remorin family protein
AT5G27030	TPR3	-26.56	68.03	Topless-related protein 3
38/45oC				
AT1G32920		327.62	-711.85	hypothetical protein (involve in:ethylene-activated signalling pathway; jasmonic acid biosynthetic process; respone to chitin, fungus, jasmonic acid and wonding)
AT1G35140	PHI-1	13.06	-29.33	Phosphate-responsive 1-like protein (involve in: response to hypoxia, and response to mechanical stimulus)
AT1G66160	CMPG1	31.51	-43.28	ubiquitin-protein ligase(involve in: intracellular signal transduction, proline transport, protein ubiquitionation, respiratory burst involved in defense response and response to chitin)
AT2G35930	PUB23	23.35	-45.53	E3 ubiquitin-protein ligase (involve in defene response, jasmonic acid mediated signaling pathway, response to chitin, water deprivation,and systemic acquired resistance, salicylic acid mediated signaling pathway, and more, see NCBI)
AT3G02840		22.57	-78.3	hypothetical protein(involve in: defense response by callose deposition, defense response to fungus,thylene biosynthetic process,response to chitin,to other organism and to ozon, and more, see NCBI)
AT3G10930		43.03	-72.59	hypothetical protein(involve in: ethylene biosynthetic process, response to chitin, response to mechanical stimulus,and response to wounding)
AT3G19680		55.44	-50.4	hypothetical protein
AT3G44260		140.11	-410.55	putative CCR4-associated factor 1(involve in: defense response to bacterium and insect,ethylene biosynthetic process,ethylene-activated signalling pathway;response to chitin, response to mechanical stimulus, response to wounding)
AT3G57450		96.91	-377.03	hypothetical protein (involve in: abscisic acid-activaed signaling pathway, jasmonic acid biosynthetic process, response to chitin, fungus, jasmonic acid and wounding)
AT4G24570	DIC2	73.46	-243.15	dicarboxylate carrier 2 (involve in: ethylene biosynthetic process, response to chitin, response to mechanical stimulus, response to wounding, and transport, see more in NCBI)
AT4G29780		41.84	-222.44	hypothetical protein (involve in: ethylene-activated signalling pathway, response to chitin, response to mechanical stimulus and response to wounding)
AT5G15350	ENODL17	31.98	-50.4	early nodulin-like protein 17
AT5G37770	TCH2	171.45	-430.15	calcium-binding protein CML24 (involve in: innate immune response, response to abscisic acid,absence of light,auxin,cold,heat, hydrogen peroxide, mechanical stimulus, metal ion, wounding and more in NCBI)
AT5G42380	CML37	101.43	-175.67	calcium-binding protein CML37 (involve in: defense response by callose deposition, ethylene biosynthetic process, heat acclimation and response to ozone)
AT5G45340	CYP707A3	15.44	-38.05	abscisic acid 8'-hydroxylase 3 (involve in: abscisic acid catabolic process, oxidation-reduction process, response to chitin, to read or far red light and to water deprivation)
AT5G54380	THE1	14.31	-12.69	receptor-like protein kinase THESEUS 1 (involve in: response to brassinosteroid)
AT5G57560	TCH4	144.71	-463.39	xyloglucan endotransglucosylase/hydrolase protein 22 (involve in: response to auxin, to brassinosteroid, to chitin, to cold, to heat, to mechanical stimulus, to wounding, and more in NCBI)
AT1G02400	GA2OX6	26.26	-51.06	gibberellin 2-oxidase 6
AT1G18300	NUDT4	62.94	-189.99	nudix hydrolase 4

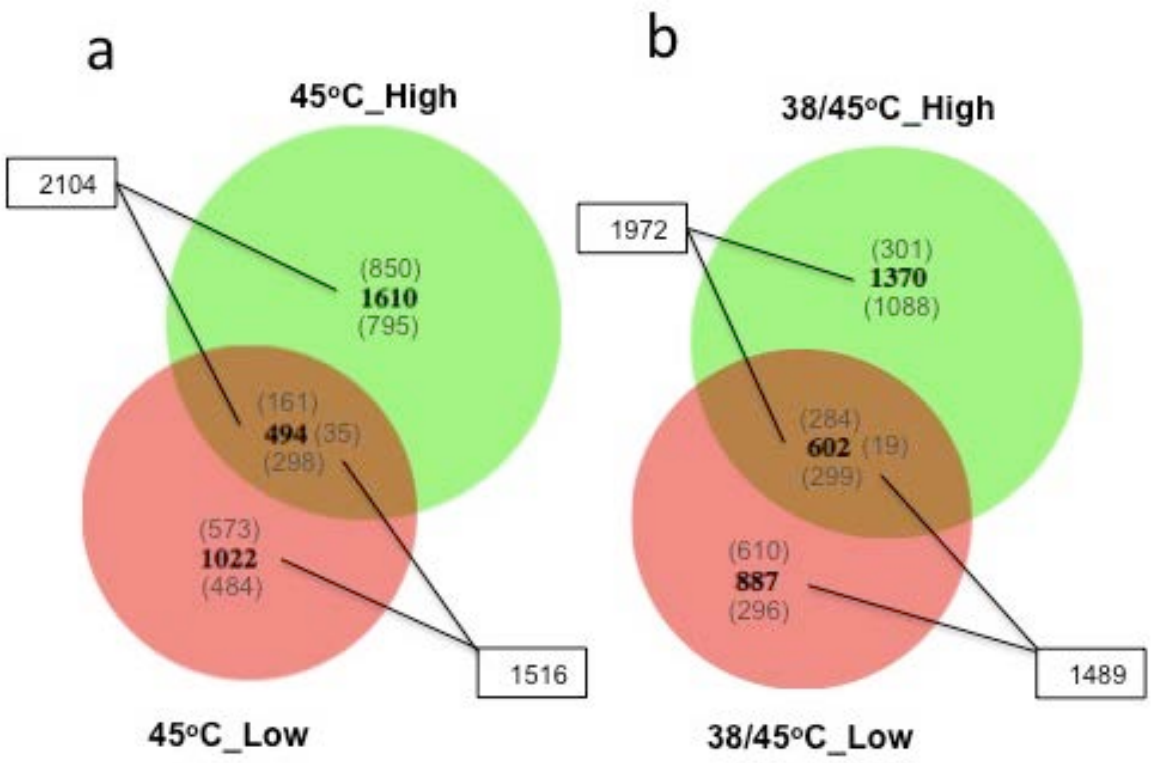


Figure 20. Venn diagrams comparing the number of DE (significantly differentially expressed) genes for the indicated treatment and elevation groups. In all cases DE genes are those differing significantly in expression compared to the same genes expressed in the corresponding 22°C control group. Bold numbers indicate total number of genes showing changed expression. Numbers in parentheses above the bold number indicate up-regulated genes and numbers below the bold indicate those that are down-regulated. The area of overlap of the two circles indicates the proportion of DE that are shared between the treatment and elevation groups. In the area of overlap, numbers in parentheses to the right of the bold numbers indicate DE genes that were shared but with directions of change between the compared groups, e.g. up-regulated in one group but down-regulated in the other group.

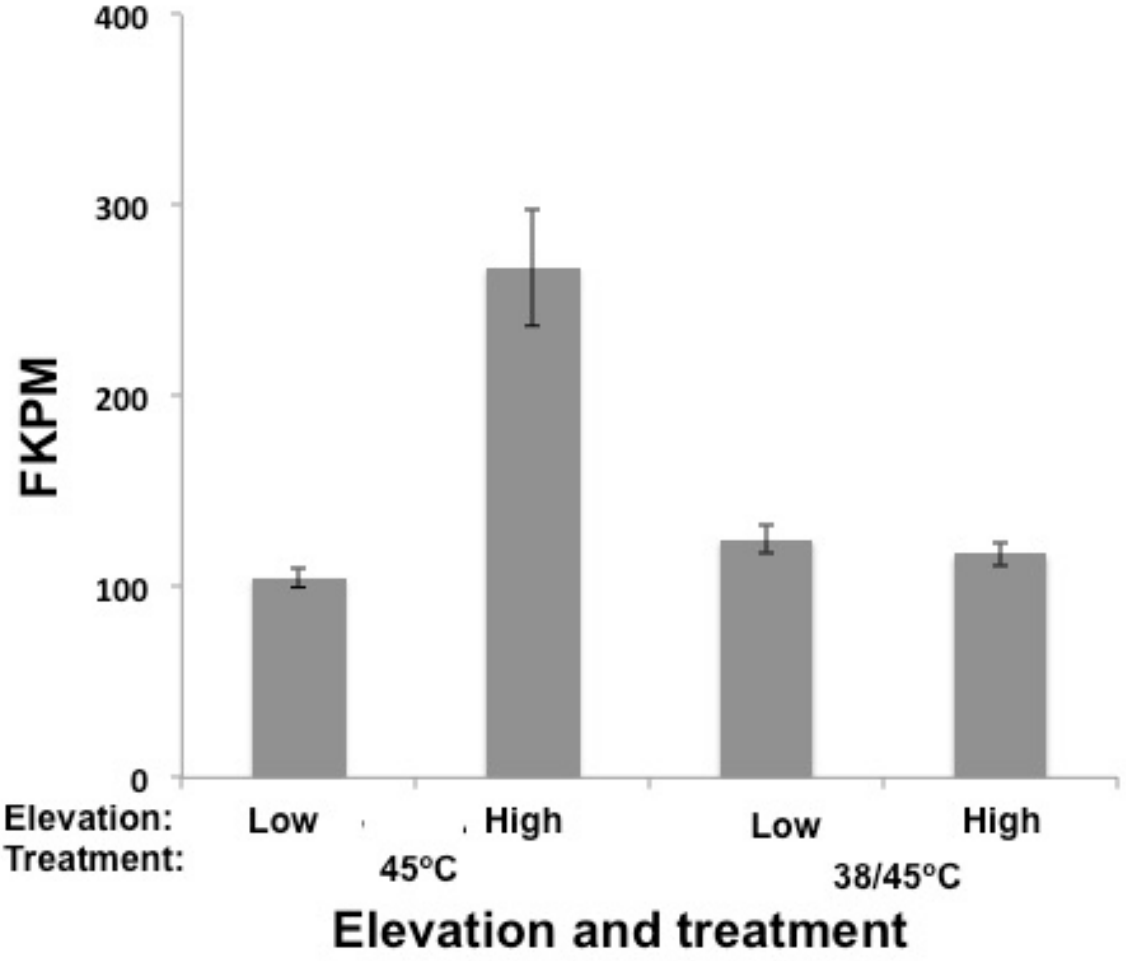


Figure 21. The difference in magnitude of the normalized fold change, FKPM, of the DE genes. The data showed are means of FKPM value for each treatment elevation pair. Error bars are standard errors.

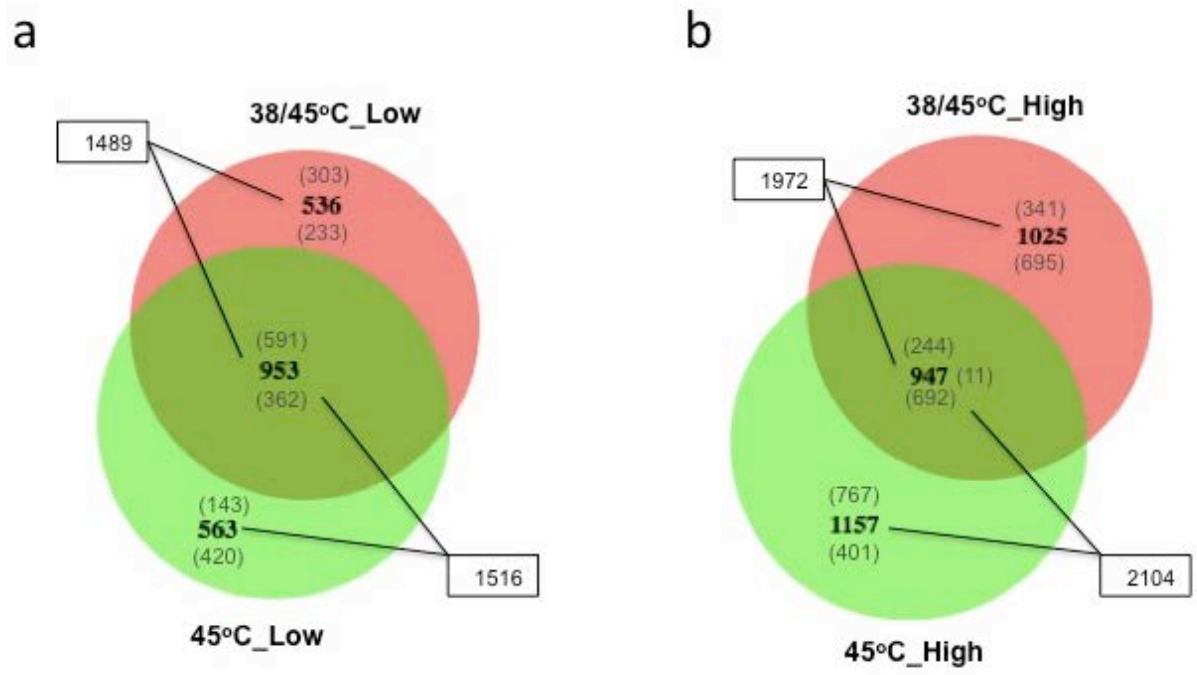


Figure 22. Venn diagrams comparing the number of DE (significantly differentially expressed) genes for the indicated treatment and elevation groups. In all cases DE genes are those differing significantly in expression compared to the same genes expressed in the corresponding 22ºC control group. Bold numbers indicate total number of genes showing changed expression. Numbers in parentheses above the bold number indicate up-regulated genes and numbers below the bold indicate those that are down-regulated. The area of overlap of the two circles indicates the proportion of DE that are shared between the treatment and elevation groups. In the area of overlap, numbers in parentheses to the right of the bold numbers indicate DE genes that were shared but with directions of change between the compared groups, e.g. up-regulated in one group but down-regulated in the other group.

7.0 WHOLE GENOME SEQUENCING IDENTIFIES GENES INVOLVED IN LOCAL ADAPTATION IN *ARABIDOPSIS THALIANA*

7.1 INTRODUCTION

Arabidopsis thaliana is a native annual plant in Europe and central Asia, and now is dispersed worldwide (Al-Shehbaz and O'Kane 2002, Koornneef et al. 2004). *Arabidopsis thaliana* has higher polymorphism in gene groups interacting with biotic stresses, compared to other species (Clark et al. 2007). It is an ideal model species for studying ecological evolution and local adaptation.

Functional information is encoded in the DNA sequences. At the population level, *Arabidopsis thaliana* evolve differently depending on conditions in their local environment. Genetic differences between populations can be due to selection for local adaptation, especially if these patterns are replicated across similar environments (Savolainen et al. 2013). Identifying genes that are directly responsible for adaptive response to local climates is essential to uncover local adaptation at the genomic level. A 1001 Genomes Project has provided a foundation for genetic variation in 1001 accessions (Ossowski et al. 2008, Cao et al. 2011, Schneeberger et al. 2011, Long et al. 2013, Schmitz et al. 2013). Populations from extreme climates have evolved differentiated genome sequences, and this genomic signature has been revealed in the non-synonymous mutations in genes specific to the extreme conditions, such as hypoxic stress (Gou

et al. 2014). To detect signatures of local adaptation, one method used with whole genome sequencing is to detect the population differentiation through comparisons of the Wright fixation index (Fst) across the genome (Savolainen et al. 2013).

Selective sweep analysis is a common used method to detect signatures of local adaptation using the Wright fixation index (Fst). A selective sweep is the reduction or elimination of variation in neighboring loci with the fixation of an advantageous mutation (Ihle et al. 2006). A strong selective sweep results in a large reduction of the total genetic variation in that chromosome region. Thus by performing selective sweep analysis among populations along an elevation gradient, we can uncover candidate genes in adapting to specific climate conditions.

Our study site, the Iberian Peninsula in northeastern Spain, is one of the six main geographic regions of *Arabidopsis thaliana*'s native range (Cao et al. 2011). In the 1001 genome project, although some accessions of our study site have been included, only one genotype from one population (Vie-0) was sequenced (Cao et al. 2011). Our 16 populations are thought to have originated from a common ancestor (Pico et al. 2008), providing a simplified model to test the genotype by environment interaction. Especially, Cao et al. (2011) also showed that populations from our region and North Africa had the most region-specific and accession-specific SNPs (single nucleotide polymorphism) compared to the other 6 regions.

In this study, we are interested in understanding the functional basis of genetic diversity and population structure for populations arrayed along an elevation gradient. These populations have been described phenotypically (Montesinos-Navarro et al. 2009, Montesinos-Navarro et al. 2011, Montesinos-Navarro et al. 2012, Wolfe and Tonsor 2014, Zhang et al. 2014, Zhang et al. 2015a, Zhang et al. 2015b). In summary, the populations exhibit clinal variation in seed traits, life history, photosynthetic physiology, and stress responses. In this study, we used re-

sequencing to generate full genome sequences for 62 genetic lines from the 16 populations in the citations listed above. After mapping the sequences, we firstly called the SNPs from all samples. Using the filtered SNPs, we then looked at genetic diversity and population structure using principle component analysis (PCA), phylogenetic tree analysis, and population structure analysis. Next we divided the populations into three elevation groups based on structure analysis and performed selective sweep analysis to identify candidate genes as signatures of local adaptation. Our study identified signatures of selection at low, medium and high elevation plants across an elevation gradient. Our study shed light on current studies in ecological genomics and local adaptation.

7.2 MATERIALS AND METHODS

7.2.1 Plant samples and growth conditions

Plant lineages were collected from NE Spain as seeds (Montesinos *et al.* 2009; Montesinos-Navarro *et al.* 2011, 2012; Wolfe & Tonsor 2014) and grown for at least three generations in common controlled environmental conditions to remove any maternal environmental variance that might otherwise have carried over from the field. The geographic locations and elevation information of these populations can be seen in Fig. 23 (adapted from Fig.1 in Montesinos-Navarro *et al.* 2001). Seeds were germinated and maintained at 22°C for 3 weeks (16 hrs light/8 hrs dark, 200 μM $m^{-2}s^{-1}$) after 5-day stratification at 5°C in the dark. All plants from which samples were taken were grown simultaneously in a randomized block design in our Conviron PGW36 controlled environment growth chambers (http://www.conviron.com) at the University

of Pittsburgh. Leaf samples were then collected. The most recently fully expanded leaves were collected prior to signs of leaf senescence, frozen in liquid nitrogen and stored in a -80C freezer, then lyophilized prior to DNA extraction.

7.2.2 DNA extraction, library preparation and sequencing

DNAs were extracted using MasterPure Plant Leaf DNA Purification Kit (Epicentre, Cat. Nos. MPP92010 and MPP92100) following the manual. DNAs were quantified using Qubit 3.0 Fluorometer (Life technologies). Libraries were prepared using Nextera DNA Library Preparation Kit (Illumina) according the Nextera DNA sample preparation protocol with modification. The quality and quantity of the DNA libraries was evaluated with a Bio-analyzer at the Genomics Research Core, University of Pittsburgh. Sequencing was done using Illumina HiSeq 2500 150bp paired-end rapid run at Duke University. We conducted two runs, one for 28 samples, and the second run for 36 samples. Samples were randomly assigned into each lane. A Miseq was performed to test the quality of the libraries before scheduling for Hiseq sequencing.

7.2.3 Sequence mapping

For the 62 genetic lines for which sequencing was effective, sequencing data was aligned with the reference sequence through BWA Software (parameters: mem -t 4 -k 32 -M) (Li and Durbin 2009), the mapping rate and coverage was determined according to the alignment results (see Table 28). The duplicates were removed by SAMTOOLS (parameters: rmdup) (Li et al. 2009). The mapping rates of samples reflected the similarity of sample and reference genome, the coverage reflected the equality and homology with the reference genome.

7.2.4 SNP detection and annotation

SNP (single nucleotide polymorphisms) mainly refers to the DNA sequence polymorphism in genomic level caused by single nucleotide mutation, including single base conversion and transversion. SAMTOOLS (mpileup -m 2 -F 0.002 -d 1000) was used to detect SNPs at population scale (Li 2011). To decrease the SNP error rate, the following standards were used to filter SNPs: (1) the reads for each SNP were more than 4, less than 1000; (2) the quality of SNPs was more than 20.

7.2.5 Population genetic polymorphism and population structure

We performed principal component analysis (PCA), population structure analysis and phylogenetic tree analysis based on the population scale SNPs. We also performed a LD decay analysis to look at the linkage disequilibrium decay across the genome for all 16 populations used in our study.

Principal component analysis (PCA) was performed based on SNP difference between individual genome. PCA was performed with EIGENSOFT4.2 (Patterson et al. 2006). We obtained the first three epigenvectors from the covariance matrix using R (Team 2014).

Population structure analysis helps understand the evolutionary process. Population genetic structure was analyzed Using Frappe. To explore the possible individual convergence, we also looked the population structure from grouping value K = 2 to K = 5. Frappe was developed in the Tang lab, Stanford (http://med.stanford.edu/tanglab/software/frappe.html). Based on analysis from ADMIXTURE (Alexander et al. 2009), which uses maximum likelihood estimation from multilocus SNPs, we decided that the optimum grouping value is K = 3.

A phylogenetic tree can describe the evolutionary relationships of different lineages among the studied populations. After the SNP detection, the individual SNPs can be used to calculate the genetic distance between populations. MEGA4.0 software was used to calculate the distance matrix, and on this basis, a phylogenetic tree was constructed by the neighbor-joining method (http://www.megasoftware.net/mega4/mega.html). Guide values (bootstrap values) were resampled more than 1,000 times.

Linkage disequilibrium (LD) decay analysis were calculated using Haploview (Barrett et al. 2005). The squared correlation (r^2) between the any two loci was calculated in a 500kb window and averaged across the whole genome.

7.2.6 Selective sweep analysis

Based on the grouping information from population structure analysis, we divided the sixteen populations into three elevation categories: Very High (PAN, VIE), High/Middle (ALE, PAL, VDM, BIS, MUR) and Low (BAR, RAB, ARB, SPE, PIN, HOR). We excluded POB, COC in this analysis because of their relatively higher genetic heterogeneity than other populations; they appear to represent admixtures of the High/Middle and Low genetic groups. We also excluded population BOS because of its substantial differentiation from the other 15 populations, and we will discuss the reason for excluding this population in detail later in the results.

To identify unique SNPs involved in local adaptation, we performed selective sweep analysis. Selective sweep analysis was performed with SweeD software. We performed pairwise comparison among the three groups. To detect unique SNPs involved in adaptation to the very high elevation climate conditions, we firstly contrasted Very High vs. High/Middle and Very High vs. Low separately to identify SNPs only found in Very High group. Then we chose the

SNPs that existed for Very High group in both comparisons as unique SNPs in Very High elevation adaptation. Similarly we also identified unique SNPs involved in adaptation to Low and High/Middle groups. This analysis is calculated in 5kb windows sliding in 1kb steps. Data points marked with red are suggested to be selective regions (corresponding to the 5% values of Fst and Pi ratio). The functions of the identified SNPs were further explored the GO enrichment and KEGG enrichment.

7.3 RESULTS

7.3.1 Sequencing evaluation and SNP detection

We obtained 850M total reads, with average 96% of the reads were mapped to the genome. An average coverage of 13 fold was identified (Table 12).

We detected a total of 2604311 SNPs (Table 13). 57.4% of SNPs were transitions, 42.6% were transversions, with a transition/transversion ratio of 1.344 (Table 13). Among these SNPs, we have 333348 upstream SNPs and 266840 downstream SNPs. We also detected 9173 stop gain and 584 stop loss because of mutation. 238872 synonymous and 278251 non-synonymous SNPs were also detected.

7.3.2 Genetic diversity and population structure

To understand the genetic relationship among the 16 populations, we firstly performed a principal component analysis (PCA) with all the SNPs (Fig. 24). Population BOS was

significantly different from the other 15 populations using principal component 1 and 2 (PC1 and PC2). When PC3 was added, another two distinct groups were identified. One group had population VIE and PAN, which are the populations from the two highest elevation sites. The other group included all the other 13 populations. Within the 13 populations group, we can also divide populations into two sub-groups, a low elevation group and high & middle elevation group (Fig. 24). To summarize, the PCA with three eigenvectors divided our samples into four groups, three groups were in accordance with the elevation grouping, with one population BOS separated from all the rest.

To further examine the genetic relationships among the 16 populations, we constructed a relatedness tree using the neighbor-joining (NJ) method. Population BOS was firstly separated from the remaining populations. The next separated population was RAB. After that, the remaining 14 populations firstly divided into two clades: one purely for low elevation populations, and the second clade can be further divided into three groups: very high, high and middle elevation populations (Fig. 25). This figure also showed a closer relationship between middle elevation and high elevation genetic lines than for low elevation lines, which is in accordance with PCA analysis (Fig. 24). The very high elevation lines were separated from the high elevation lines. Most of the lines within a population clustered together, except three lines, ARB10, MUR16 and ALE16. Although they do not cluster within their population group, the three lines still cluster within the high elevation group (Fig. 25).

To further explore the possible genetic mixture among the populations, we performed a population structure analysis to look at the genetic composition among these populations (Fig. 26). We showed the genetic structure among populations from K = 2 to K = 5. With K = 2, the populations were roughly divided into groups of populations with pure genetic information, and

several populations with mixed genetic information from the two pure groups. With K = 3, the two highest elevations, PAN and VIE, were separated from group of pure high elevation genetic information. When K increased to 4, population BOS was separated from the group of pure low elevation genetic information. When K = 5, two low elevation populations, HOR and PIN, were separated from the low elevation group. Our analysis also estimated the optimal K value is 3, supporting the very high, high and low elevation classification.

Putting the three analyses together, the 15 populations (with BOS excluded) can be divided into four groups: very high, high, middle and low elevation. However, sometimes high and middle elevation groups tend to mix with each other, indicating genetic admixture between the two groups. Low elevation plants shared a relatively distant relationship with middle elevation plants, compared with high and very high elevation plants.

It was not surprising to see population BOS acted so differently from the rest 15 populations. We have previously seen that BOS showed very different patterns in the phenotypic traits in the lab (e.g. Montesinos-Navarro et al. 2011). Our collection site was adjacent to a truck repair facility in the village of Bos Ost, which lies along a major transport route across the Pyrenees. Thus it is possible this population was transported by truck from another phylogeographic region. Because of the difference, we have excluded this population from the remainder of this study. The whole genome sequencing data further confirmed its distant relationship with the other 15 populations. We will not discuss the BOS population further in this paper.

Finally, the differences among the 16 populations were also shown in the linkage disequilibrium (LD) patterns (Fig. 27). For example, several populations, HOR, MUR and ALE, showed much higher LD decay level than the rest of populations. Another two populations, COC

and VDM, showed much lower LD decay level than the others. We also saw that the two populations in the Very High group, VIE and PAN, showed very flat LD decay across the genome.

7.3.3 Selective sweep analysis for signatures of local adaptation

When contrasted Very high vs. High/middle group, we discovered 8 SNPs that only existed in the Very High elevation group. When we contrasted Very High vs. Low group, we found 9 SNPs that are unique for Very High elevation group (Table 28). Only one unique SNP, AT1G64380.1, in the ethylene-responsive transcription factor ERF061, was found in Very High group. ERF061 encodes a member of the DREB subfamily A-6 of the ERF/AP2 transcription factor family (Berardini et al. 2015). ERF061 is involved in the ethylene signaling pathway and in transcription regulation.

Similarly, we found 174 unique SNPs for the Low elevation group when we contrasted Low vs. Very High groups, and we found 618 unique SNPs for the Low group when compared Low vs. High/Middle group. Among them, only four SNPs were unique in low elevations when compared with the other two groups. The four candidate SNPs involved in adaptation to low elevation climate are: AT2G32670.1, in Vesicle-associated membrane protein 725 VAMP725, AT2G32680.1, in receptor like protein 23 RLP23, and AT5G05750.1, in DNAJ heat shock N-terminal domain-containing protein, as well as AT5G05760.1, in Syntaxin-31 SYP31. VAMP725 is involved in protein transport and vesicle fusion, RLP23 is involved in signal transduction and defense response, DNAJ heat shock N-terminal domain-containing protein is involved in protein folding, and SYP31 is involved in intracellular protein transport, vesicle docking and vesicle fusion. AT2G32670.1 and AT2G32680.1 are next to each other on

chromosome 2, AT5G05750.1 and AT5G05760.1 are next to each other on chromosome 5 (Berardini et al. 2015).

When we compared High/Middle vs. Very High and High/Middle vs. Low separately, we found 154 and 453 SNPs unique to High/Middle group. Among them, only one SNP, AT4G23190.1, in Cysteine-rich receptor-like protein kinase 11 CRK11, was unique to High/Middle group in the three groups. CRK11 encodes putative receptor-like protein kinase that is induced by the soil-borne vascular bacteria, Ralstonia solanacearum (Berardini et al. 2015).

7.4 DISCUSSION

Genomic sequences can reveal the evolutionary history of *Arabidopsis thaliana*. Understanding how genomes respond to the environment is key to uncovering the genetic basis of local adaptation. In this study, 64 genetic lines, from 16 populations, along an elevation gradient, were collected and subjected to whole genome re-sequencing and sequence analysis. We obtained an average of 13-fold coverage for each sample and a total of 2,604,311 SNPs. The 16 populations can be further divided into three groups based on analysis performed using the SNPs. The classification is in accordance with classification based on elevation of origin. Populations in the very high elevation are closer to populations in the high and middle elevation, compared with populations from the low elevation. Further analysis indicated different linkage disequilibrium (LD) patterns among these populations. We also detected one SNP on chromosome 1 in adaptation to very high elevation, four SNPs, two on chromosome 2 and two on chromosome 5, in adaptation to low elevation, and one SNP in adaptation to high/middle elevation.

Previously work on nine of the 16 populations, using fewer SNPs, high elevation populations were shown to be genetically less diverse than low elevation populations. Middle elevation populations also exhibited low genetic diversity (Gomaa et al. 2011). This is also true for our data analysis. High/Middle elevation populations tend to look like more like the two very high elevation populations (Fig. 24, Fig. 25, Fig. 26).

Selective sweeps at the species level are very rare. Currently only one 0.5M deletion on chromosome 1 from 100 Arabidopsis genetic lines was found, confirmed by extremely low allele frequencies from two studies (Clark et al. 2007, Cao et al. 2011). Within species, selective sweeps can increase differentiation at population level when populations undergo diversifying selection (Nielsen 2005, Gou et al. 2014). We discovered six unique SNPs, that is six candidate genes, involved in adaptation to environment conditions in one of the three elevation groups. This kind of partial selective sweeps that only shows in some populations within species can be used as signature of local adaptation(Savolainen et al. 2013).

The ERF/AP2 family, to which ERF061 belongs, contains a plant-specific transcription factor that activates the expression of abiotic stress-responsive genes. Previous studies, including our previous whole transcriptome sequencing analysis (Zhang et al. under review), have shown several other ERF/AP2 family genes, such as DREB2A, DREB2B, ETR1 and EIN2, that are known to be involved in stress response (Kotak et al. 2007, Ahuja et al. 2010, Qu et al. 2013).

The LD pattern we observed indicates the decay in relatedness with increasing sequence distance (Nordborg and Tavaré 2002, Flint-Garcia et al. 2003). Regional differences in LD decay pattern might relate with the size of the geographic area, with smaller area having lower LD pattern (Cao et al. 2011). In our study, we have not put the size of geographic region of these populations into consideration before, and maybe it is worth to have a look.

Our analysis of the 64 whole genome sequences can be a first step to explore the genetic signature of local adaptation. To further understand the genetic basis under each particular adaptive phenotypic trait, studies need to link the genetic variation and phenotypic variation together (Savolainen et al. 2013). Also, adaptive responses can rise from standing variation or new mutation. However, the signal of standing variation may be weaker, compared with new mutations (Savolainen et al. 2013). To uncover whether a selective sweep is a standing variation or new mutation, we need to connect our genome data with the other published genome data, such as sequences from 1001 genome project. Our study site, Iberian Peninsula, is a hotspot for plant biogeographic research (Comes 2004). Filling our *Arabidopsis thaliana* sequences into the sequences database can contribute greatly into the research community on agriculture, horticulture and evolutionary ecology.

Table 12. Alignment summary of whole genome sequencing on the 63 samples.

Sample Name	Elevation (m)	Mapped Reads	Total reads	Mapping rate (%)	Average depth	Coverage>=1X	Coverage >=4X
ALE10	1163	17162955	17529380	97.91	16.08	93.1	85
ALE12	1163	16565358	17022684	97.31	16.43	93.52	89.29
ALE16	1163	3389220	3537816	95.8	4.4	72.09	16.75
ALE4	1163	24397831	24998582	97.6	25.61	94.23	92.39
ARB10	440	10888541	11288012	96.46	9.48	90.71	69.02
ARB3	440	18230764	18821758	96.86	16.92	93.96	86.82
ARB6	440	16322722	16930162	96.41	13.47	93.33	84.24
ARB8	440	13767400	14165888	97.19	14.26	93.74	86.65
BAR11	340	6129438	6485748	94.51	5.4	74.2	31.81
BAR3	340	10754563	11152780	96.43	10.61	92.81	81.15
BAR4	340	15185024	15524966	97.81	15.18	94.19	87.71
BAR9	340	12352230	12946446	95.41	9.68	89.45	64.21
BIS11	1397	2325639	2400012	96.9	3.92	54.41	9.93
BIS16	1397	10790867	11084672	97.35	11.88	93.77	83.17
BIS20	1397	11172679	11482666	97.3	11.91	93.96	83.16
BIS8	1397	10821208	11225944	96.39	10.13	91.18	69.14
BOS10	719	22460461	22985602	97.72	21.52	94.66	92.53
BOS5	719	15939176	16320842	97.66	15.39	94.25	86.71
BOS6	719	12641240	12900992	97.99	12.82	93.86	84.43
BOS9	719	17936448	18317682	97.92	18.36	94.69	92.09
COC14	519	16637733	17112844	97.22	15.51	93.75	86
COC17	519	11533701	11832378	97.48	12.52	93.95	84.36
COC19	519	13108992	13491032	97.17	13.78	93.82	87.74
COC7	519	6953956	7377378	94.26	7.26	87.83	52.02
HOR16	351	17685309	18164022	97.36	19.41	94.28	91.34
HOR4	351	14028908	14525536	96.58	13.54	93.73	86.76
HOR6	351	16455836	17003402	96.78	16.88	94.32	91.49
HOR7	351	12750465	13293150	95.92	11.72	93.23	82.69
MUR15	836	16436590	16895250	97.29	15.85	93.14	84.95
MUR16	836	11301169	11654396	96.97	11.75	95.36	83.67
MUR17	836	13037301	13701900	95.15	11.61	91.65	76.57
MUR12	836	11179547	11567732	96.64	11.35	92.96	83.8
PAL12	1491	16443702	17006354	96.69	15.67	94.35	89.98

PAL16	1491	3672514	3772658	97.35	4.62	85.03	28.25
PAL6	1491	8079072	8288914	97.47	9.13	92.35	70.58
PAL7	1491	11186325	11539864	96.94	11.71	94.12	85.79
PAN1	1664	12167890	12443554	97.78	12.56	93.22	80.16
PAN5	1664	9942264	10308438	96.45	8.68	88.63	56.13
PAN9	1664	11743533	12124412	96.86	11.69	93.47	83.88
PIN3	109	14168172	14564322	97.28	13.97	93.85	87.86
PIN6	109	10802767	11221942	96.26	11.54	94.11	87.11
PIN7	109	12134717	12458552	97.4	12.33	93.54	84.43
PIN9	109	18334616	18672492	98.19	18.74	94.26	89.78
POB10	597	13520151	13977944	96.72	12.03	92.45	80.82
POB16	597	13124776	13637742	96.24	13.44	94.17	89.38
POB19	597	23340272	24147666	96.66	22.09	94.51	92.64
POB7	597	10861398	11153816	97.38	11.53	93.67	81.57
RAB17	110	16592715	17090032	97.09	16.63	93.82	90.65
RAB20	110	20481213	21019170	97.44	19.88	94.02	91.45
RAB4	110	4651043	4790876	97.08	5.57	81.68	35.62
RAB9	110	15048695	15535136	96.87	15.72	94.57	91.84
SPE2	332	12412851	12708404	97.67	12.93	93.57	85.36
SPE5	332	7349531	7687850	95.6	6.82	81.52	45.63
SPE6	332	18094308	18560778	97.49	18.45	94.54	91.05
SPE7	332	9113482	9379568	97.16	9.64	92.7	77.56
VDM17	912	14425461	14793870	97.51	14.44	93.71	88.02
VDM20	912	14160556	15816208	89.53	14.41	94.09	88.73
VDM9	912	10627858	10835608	98.08	11.25	92.07	73.96
VIE16	1538	13695600	14033550	97.59	14.2	94.08	85.83
VIE3	1538	20487573	20976644	97.67	18.35	93.98	88.49
VIE4	1538	12749595	13188256	96.67	12.26	93.74	84.11
VIE6	1538	14673306	15056750	97.45	15.15	94.11	90.27

Note: Average depth: The average sequence depth, which is the rate of reads number and genome size; Coverage at least 1X: The percentage of base site with more than one reads in total bases; Coverage at least 4X: The percentage of base site with more than four reads in total bases.

Table 13. Statistic results of SNP detection and annotation.

Category	Number of SNPs
Upstream	333348
Exonic Stop gain	9173
Exonic Stop loss	584
Exonic Synonymous	238872
Exonic Non-synonymous	278251
Intronic	343804
Splicing	2282
Downstream	266840
Upstream/downstream	118566
Intergenic	1023591
ts	1493621
tv	1110690
ts/tv	1.344
Het rate (%)	0.854
Total	2604311

Detailed explanation of the terms used:

1) Upstream:1 Kb upstream of the gene region.

2) Exonic: variation was in exonic region:

Stop gain: mutation of obtaining stop coden;

Stop loss: mutation of losing the stop coden;

Synonymous;

Nonsynonymous.

3) Intronic: variation was in intronic region.

4) Splicing: variation was in splicing region.

5) Downstream:1 Kb downstream of the gene region.

6) Upstream/Downstream: 1Kb sequences located in both upstream and downstream.

7) Intergenic: variation was in intergenic region.

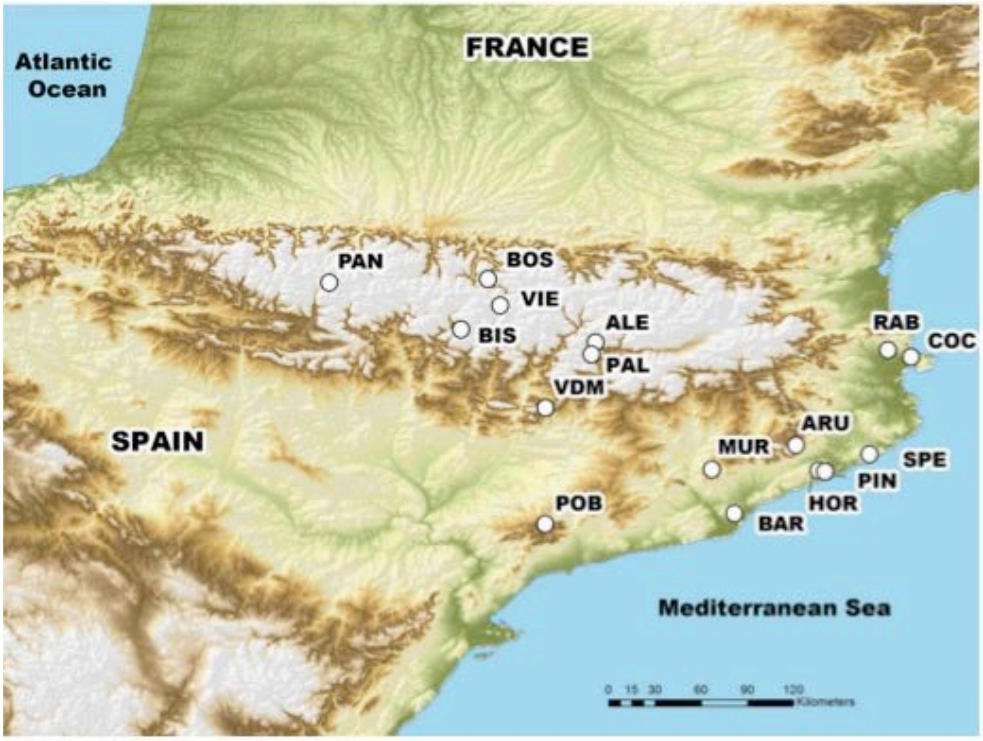
8) ts: transitions.

9) tv: transversions.

10) ts/tv: the ratio of transitions to transversions.

11) Het rate: the rate of heterozygous SNPs in genome size.

12) Total: the number of total SNP.



Population	Elavation (m)
PIN	109
RAB	110
SPE	332
BAR	340
HOR	351
ARB(ARU)	440
VIL(COC)	519
POB	597
BOS	719
MUR	836
VDM	912
ALE	1163
BIS	1397
PAL	1491
VIE	1538
PAN	1664

Figure 23. Geographic location and their elevation information of the 16 populations.

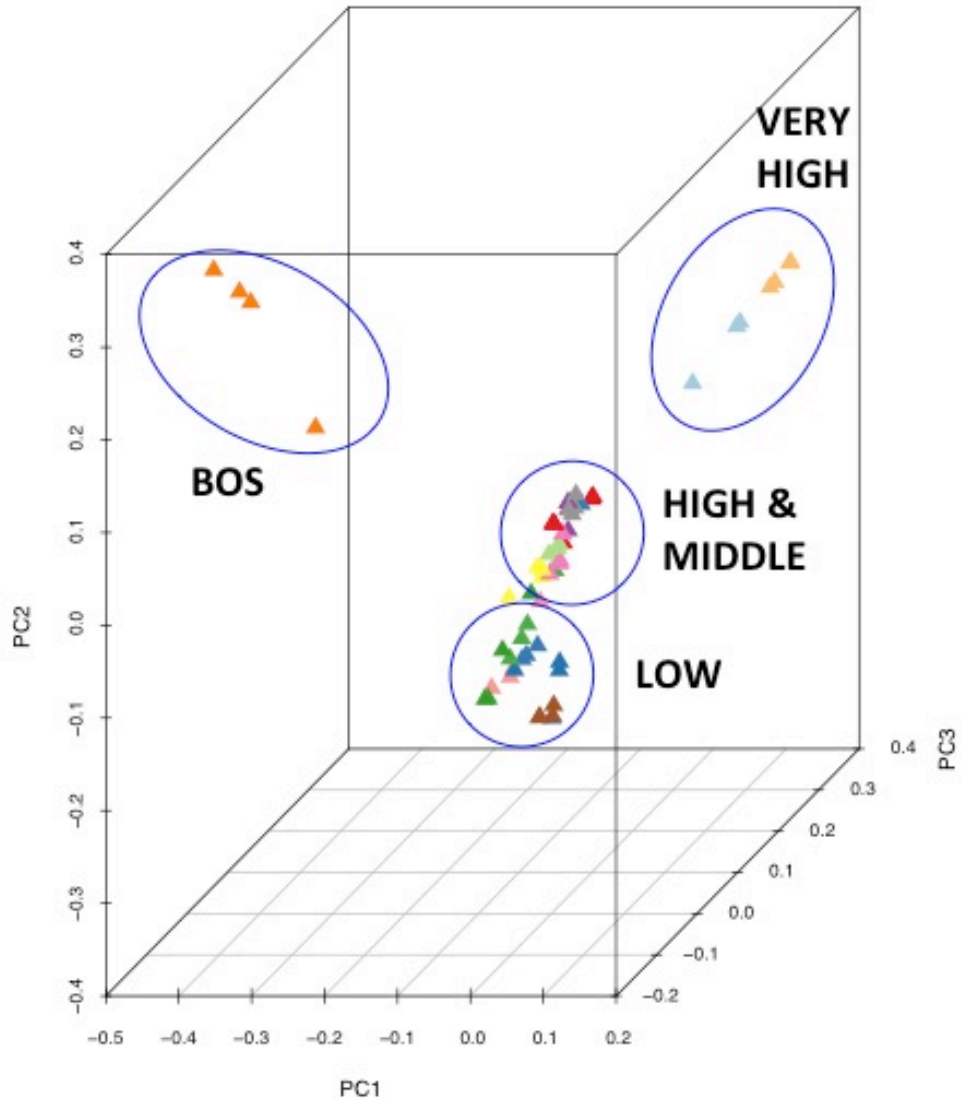
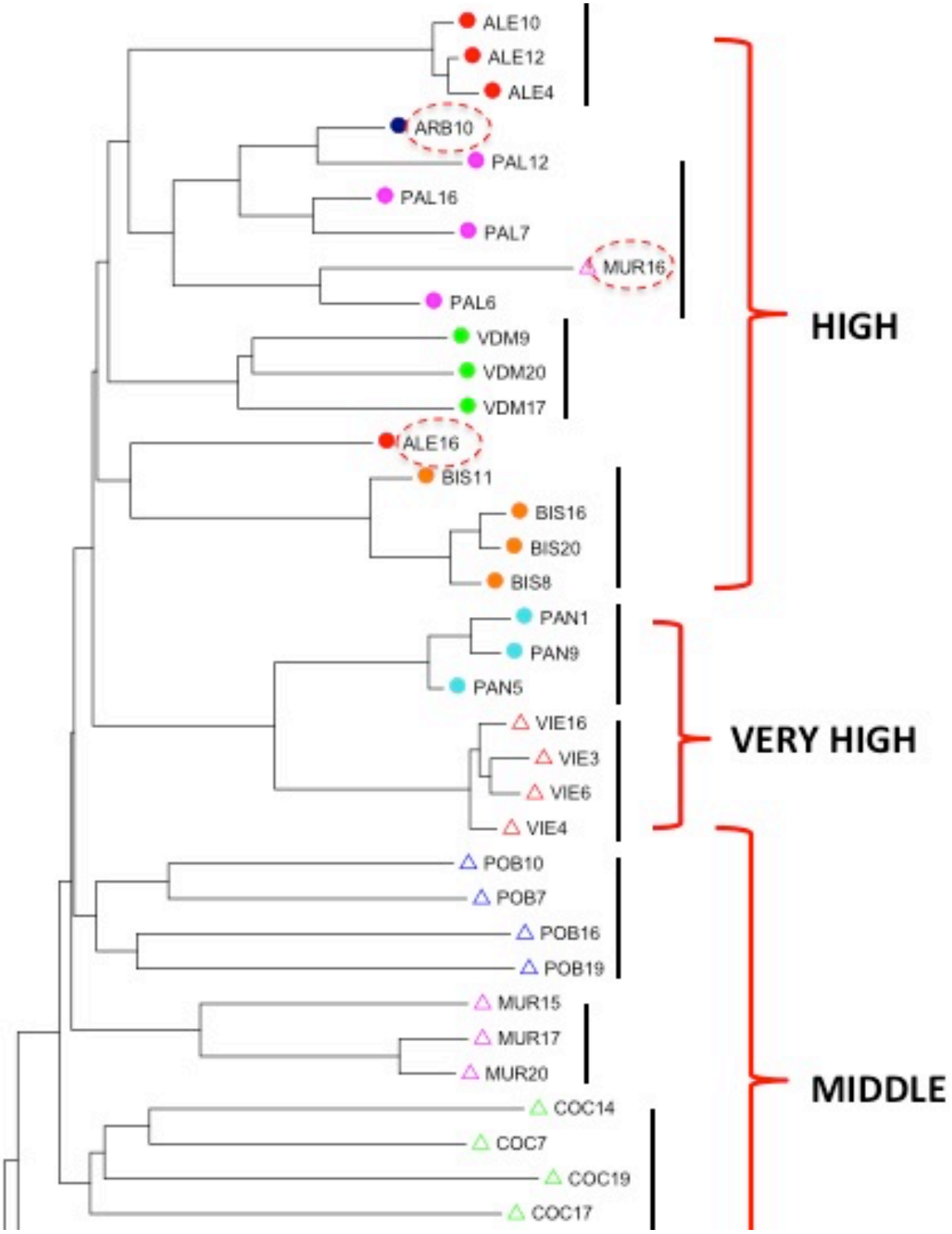


Figure 24. Principal component analysis separated the 16 populations into four groups.


ALE10
ALE12
ALE4
ARB10
PAL12
PAL16
PAL7
MUR16
PAL6
VDM9
VDM20
VDM17
ALE16
BIS11
BIS16
BIS20
BIS8
PAN1
PAN9
PAN5
VIE16
VIE3
VIE6
VIE4
POB10
POB7
POB16
POB19
MUR15
MUR17
MUR20
COC14
COC7
COC19
COC17
HIGH
VERY HIGH
MIDDLE

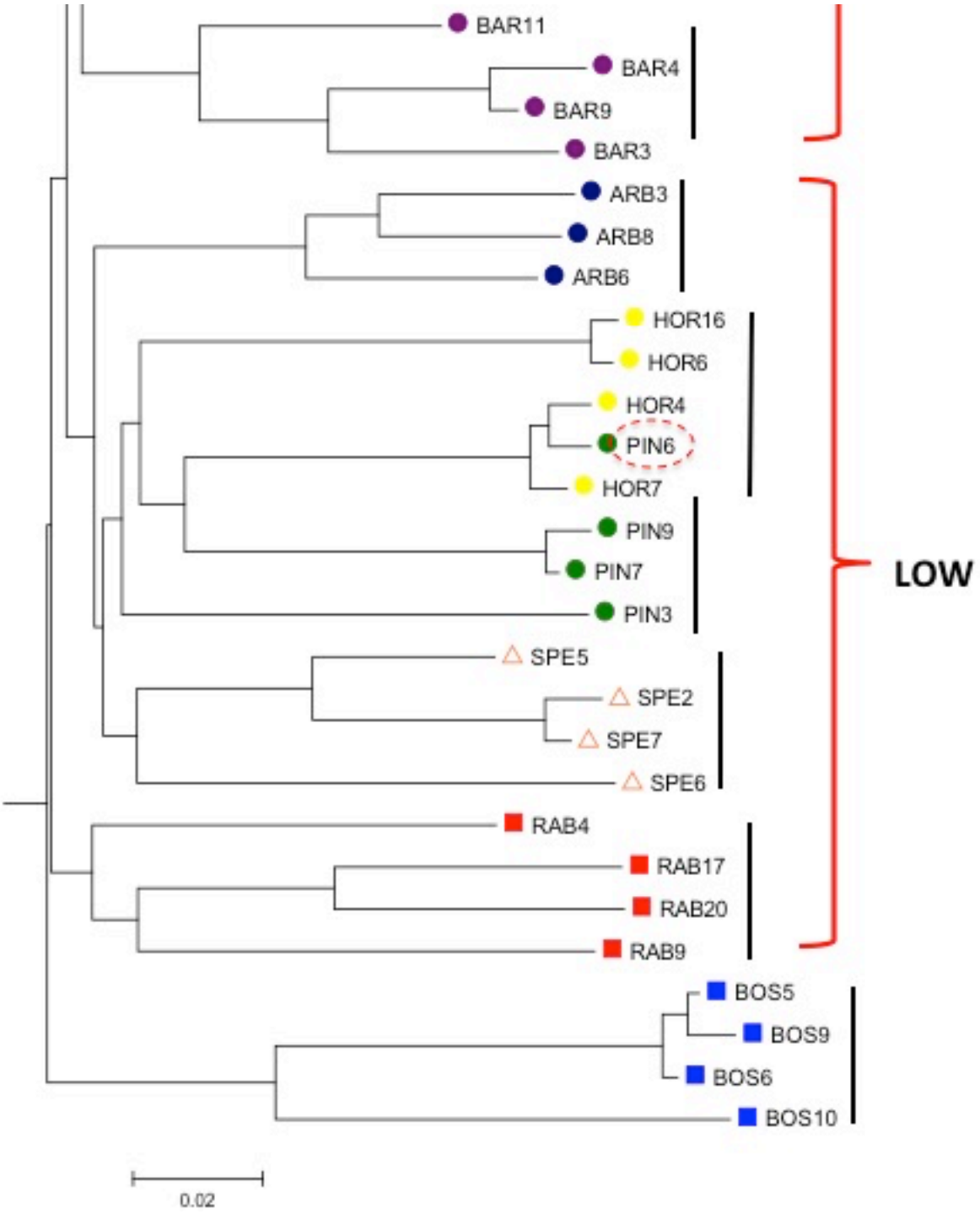


Figure 25. Phylogenetic tree based on all the SNPs using neighbor-joing (NJ) method.

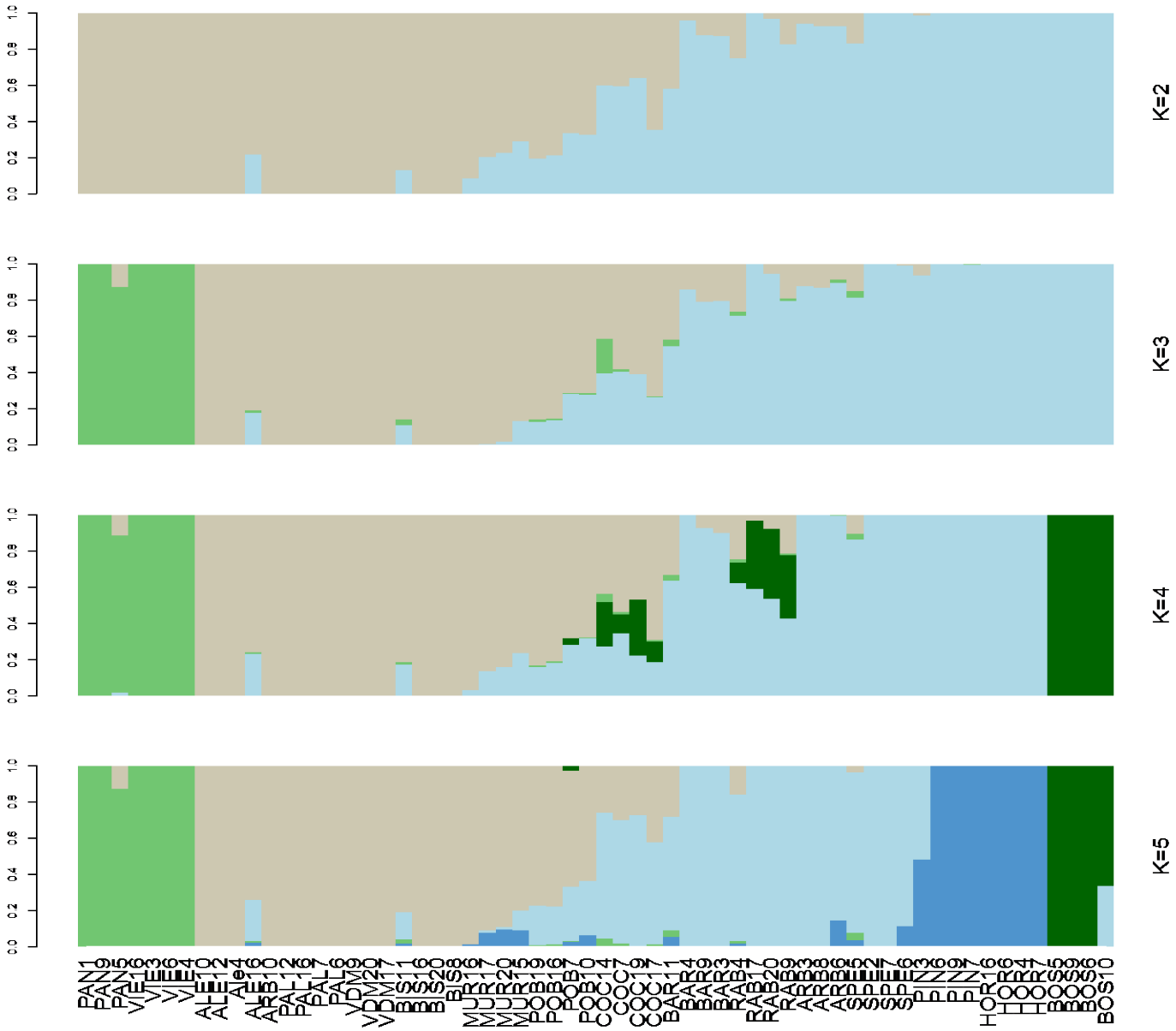


Figure 26. Population structure of the 16 populations, from K = 2 to K = 5. From left to right, the figure was organized from high to low elevation, except population HOR and BOS on the right side of the figure.

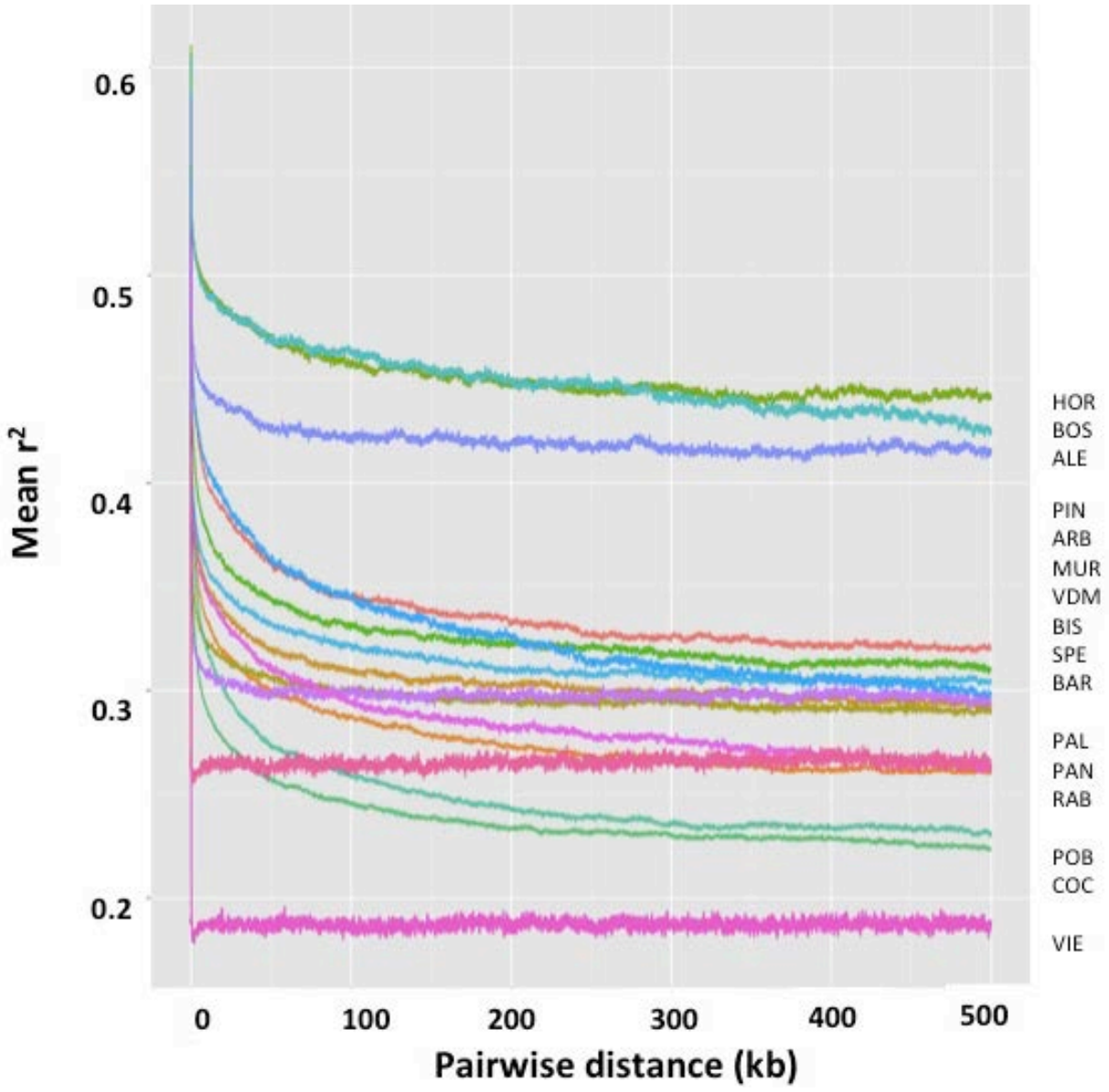


Figure 27. LD decay patterns of the 16 populations. The r^2, the squared correlation between any two loci in the genome, was calculated in 500kb window and averaged across the genome.

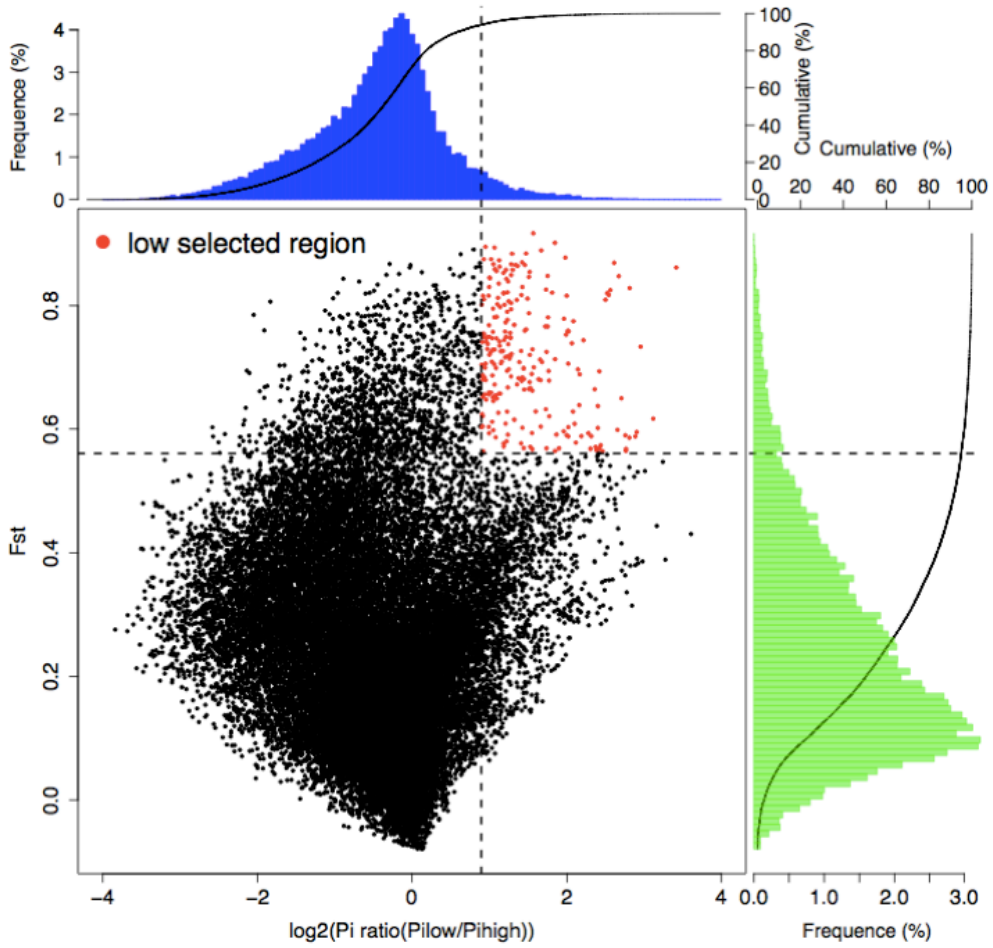


Figure 28. Selective sweep result of low elevation group comparing with very high elevation group based on Fst and Pi, as an example to show selective sweep analysis. This analysis is calculated in 5kb windows sliding in 1kb steps. Data points marked with red are suggested to be selective regions (corresponding to the 5% values of *Fst* and Pi ratio). Data points located to the left and right of the left and right vertical dashed lines, respectively (corresponding to the 5% left and right tails of the empirical ratio distribution, where the ratios are 0.57 and 1.10, respectively), and above the horizontal dashed line (the 5% right tail of the empirical F_{ST} distribution, where F_{ST} is 0.361) were identified as selected regions for low elevation group (blue points) and very high elevation group (green points), respectively. The other selective sweep analysis figures can be found in the supplementary Fig. 43 – Fig. 47.

8.0 CONCLUSION

In this dissertation, I investigated the variation and local adaptation in responses to several stressors for natural populations of *Arabidopsis thaliana* along an elevation gradient in Iberian Peninsula. Our combined results not only showed that these populations differ in their response to various stressors, such as cold, heat and pathogen infection, at a phenotypic level, but also provided a framework for exploring the underlying transcriptomic and genomic variation in stress response (Figure 29). Across all the studies, we have shown that these populations are adapted to their local climates in their stress responses. Our studies showed that species differ significantly in their allocation to avoidance and tolerance strategy in heat stress response and they also differ in the emphasis of specific avoidance or tolerance mechanisms. This study refined our ability to understand how stress responses evolve over time and to predict how species are likely to respond to changing global climates. The results are especially helpful for understanding the evolutionary history and potential of various stress response mechanisms.

This is the first study to visually show the effect/power of avoidance in stress response in plant species. The importance of avoidance deserves a re-emphasis based on the findings from our studies. We also showed that plants from high elevation favor an avoidance strategy while plants from low elevation favor a tolerance strategy, which correlates with our understanding in their local environment. In our transcriptome data, we discovered over 2100 and 1500 differentially expressed genes in high and low elevation plants, respectively, with about 500

genes being differentially expressed in both low and high elevation plants. These data deserve further exploration using gene ontology enrichment to understand their functions. These genes also provide candidates involved in stress avoidance strategy. In the genome data, we received 850M sequences data, and 2.6M SNPs, and we have just started to explore these data. These data will also potentially contribute to our understanding on stress avoidance as well as the differential allocation in avoidance and tolerance in stress response. We expect these data to fill the missing knowledge in stress response.

This dissertation also provides a more complete picture of stress response in nature across many climate regions. Climate variables are highly correlated with elevation gradient generally. In our study site, the climatic gradient from the Mediterranean shore to the high Pyrenees captures approximately 2/3 of the first principle component (PC1) of BioClim's 19 climate variables for *Arabidopsis thaliana*'s entire Eurasian distribution (Tonsor, unpublished). PC1 for the study region is highly correlated with PC1 for *Arabidopsis thaliana*'s Eurasian distribution ($r > 0.9$; Wolfe and Tonsor 2014). Because of the adaptive response to the local conditions, one thing for sure is that there is no universal solution for coping with global climate change. For future breeding and species conservation, we need to personalize the strategies depending on the details on the local environment, both abiotic and biotic conditions. Good news is that we see that our study populations have adapted to the historical stresses in their local environment. By learning from these past adaptation strategies and mechanisms, we can help them prepare better for the increasing threat from global climate change.

All organisms experience stresses, often on a daily basis. Most often plants face multiple simultaneous stresses. Plants' long-term responses to locally differing combinations and temporal patterning of stress can eventually lead to local adaptation. The adaptive response can

influence species distribution, expansion, and community composition. Populations within species but living in different climates can differentially adapt and different species living in the same climate can evolve similar traits. Understanding population differentiation in response to stresses can lead to inferences about strategies/mechanisms favored by natural selection in each particular region in stress response. Understanding how different species react similarly to common environment can help us develop universal optimal strategies to cope with stress at the community level. Studies in my dissertation support a universal stress response pathway (ROS pathway), but also show variation in the specific pathway (Hsp/Hsf pathway) evolved in response to a particular pattern of environmental stress.

All living organisms adopt avoidance and tolerance strategies in response to stresses. Understanding the specific mechanisms involved in avoidance and tolerance and their genetic basis is very crucial. Seedling plants provide us an opportunity to solely study stress tolerance. In the first three chapters (Chapter 2, 3 and 4), I explored plants' tolerance to cold, heat, and pathogen infection examining traits measureable at the seedling or cellular level. In tolerance to heat stress, two main types of pathways are involved. One is Hsp/Hsf pathway, including the up-regulation of many Hsps (heat shock proteins) and Hsfs (heat shock transcription factors); this pathway is specific to heat stress. The second pathway is the response to oxidative stress via reactive oxygen species (ROS), including many plant hormones, and this pathway is a universal stress response. I chose representative heat shock protein, Hsp101, and biochemicals, salicylic acid and camalexin, to measure the response of both Hsp/Hsf and ROS pathway in seedlings, respectively.

In Chapter 2 and Chapter 3, I tested constitutive and induced levels of salicylic acid (SA) and camalexin expression. SA is an important plant hormone in the ROS pathway whose

expression is modulated in response to stresses produced by heat, cold, and pathogen infection. Camelexin is an indole alkaloid that metabolizes form a phytoalexin that is active in suppressing a variety of plant pathogens. Its expression is modulated by heat and SA induction. In Chapter 2, I showed that the constitutive level of tissue SA declines in a geographic cline with increasing elevation. SA decreased when plants were exposed to a 44ºC heat stress. Our measure of camalexin indicated a similar decline as elevation increases. These results suggest a cline in pathogen pressure, an important biotic stressor in natural environment, as a possible explanation of the SA and camelexin cline. In Chapter 3, the induced camalexin level, after pathogen infection, was negatively correlated with the constitutive camalexin level. The camalexin accumulation was negatively related with leaf bacteria titer. These two studies together suggest that low elevation populations may experience chronic pathogen pressure, whereas high elevation plants have a more plastic response to a less predictable threat from pathogens.

In Chapter 4, I explored the variation of one key heat shock protein, Hsp101, and phenotypic variation in thermotolerance in seedlings. Hsp101 expression was significantly increased by exposure to temperatures at or above 38ºC, as expected. Hsp101 up-regulation differed by heat treatment and further differed if plants were exposed a 38ºC pre-treatment. This again was an expected result. Our novel results showed that seedling survival, post-stress root growth at 45ºC and Hsp101 expression at 42ºC were significantly correlated with the home sites' first principal component of climate variation, which mainly reflects a temperature and precipitation gradient. This study set the basis for studying the Hsp/Hsf pathway in heat stress response.

When plants grow into adult stage, both avoidance and tolerance mechanisms have been fully developed. In addition, it is in the adult (i.e. reproductively mature) stage that heat stress is

most likely to be encountered (Montesinos et al. 2009, Wolfe and Tonsor 2014). Thus in the following three Chapters (Chapter 5, Chapter 6, and Chapter 7), I expanded the heat stress response studies to include avoidance as well as tolerance. Although plants can adopt both strategies in stress response, plants along an elevation gradient could evolve differently in favoring one strategy over the other. In Chapter 5, genetic lines from low elevation sites were shown to have evolved a greater level of tolerance, by adjusting Hsps and plant hormone expression. Genetic lines from high elevation sites adopted more avoidance, by increasing transpiration rate and cooling their rosettes well below ambient temperature and well below the level of transpirational cooling achieved by the low elevation genetic lines. This is in accordance with our prediction about contrasting avoidance – tolerance strategies in adapting to their climate conditions. Low elevation plants developed rapidly increased Hsp101 and SA mechanisms when exposed to high temperature, contributing to their high temperature tolerance, as evidenced by their ability to maintain photosynthesis at high temperature. Low elevation plants also have a constitutively high leaf angle, constantly avoiding high levels of heat loading. High elevation plants, in contrast, experience heat stress occasionally and also have relatively high soil moisture availability. As a consequence high elevation plants can use high transpiration rates to avoid high tissue temperature.

In Chapter 6, I explored the differentially expressed genes between low and high elevation plants with or without a pre-acclimation (45^{o}C vs. 38/345^{o}C). High elevation plants had more differentially expressed genes than low elevation plants in both heat treatments. In 45 ^{o}C, only Hsp/Hsf pathway was activated in low elevation plants; both Hsp/Hsf and ROS pathways were activated in high elevation plants. Small Hsps had the highest magnitude of change in low elevation plants while Hsp70 and Hsp90 showed the largest magnitude of

expression change in high elevation plants. In 38/45 ºC, Hsp/Hsf and ROS pathways were activated in both low and high elevation plants. Low elevation plants showed up-regulation in all Hsps, especially small Hsps; high elevation plants showed down-regulation in all Hsps. Low elevation and high elevation also differed in gene expression response in the ROS response pathways. These differences in gene expression between low and high elevation plants indicate local adaptation in temperature stress responses in genetic lines from low and high elevations. Gene expression was relatively stable in low elevation plants in both heat treatments, especially for small Hsps. This is possibly because of the chronic stress that low elevation plants experience, leading to chronic up-regulation of small Hsps and perhaps other less well-understood pathways.

In Chapter 7, I further explored the population polymorphism and population structure of these 16 populations from our study site. In accordance with their elevation of origin, genomic data divided these populations into three groups: Very High, High/ Middle, and Low. To identify genes involved in adaptation to their specific climate conditions, we also performed pairwise comparisons among the three groups. The genetic variations that were specific to each elevation group were thus candidate genes involved in local adaptation. We found six candidate genes that might be involved in local adaptation.

The big goal is to integrate variation and local adaptation in stress response across the three levels and connect them together. For example, we already saw a consistent involvement of Hsps in all the three levels. We saw Hsp101 was up-regulated in both groups at the phenotypic level, but we only detected its up-regulation in the low elevation plants at the transcriptome level, perhaps owing to the time post-stress (immediate) in the transcriptome experiment. Similarly, we also saw only high elevation plants adopted ROS pathway genes. Our thought on

this is that low elevation plants might already be prepared for stress tolerance through consitutive up-regulation as well as unmeasured and unknown tolerance mechanisms, but high elevation plants need longer time to respond heat stress by producing heat shock factors and ROS related genes. We did see differences in specific groups of Hsps expressed between low and high elevation plants, and this might be related with the complementary or overlapping functions of the Hsps. However, we also saw mis-match in our results from distinct studies. The differentially expressed genes (2100 and 1500 for high and low elevation plants, respectively) also provide candidate genes for clarifying avoidance strategy. There are many opportunities for making use of these data to answer scientific questions.

An immediate challenge is to identify candidate genes involved in adaption to each elevation condition and to verify their function in vivo. I have identified many genes that showed differential expression at the transcriptome level, but we have thus far only looked at those genes that were previously reported to be involved in stress tolerance. We need to explore the other differentially expressed genes in the list. Especially, we need to look for genes involved in avoidance. To the best of my knowledge, there has not been enough information about genes involved in avoidance to stress. I also uncovered some candidate genes showing evidence of selection that was specific for Very High, High/Middle, Low elevation population groups in Chapter 8. We need to contrast our candidate gene list with other studies worldwide to test whether these candidate genes are specific in our study region or they are universally adaptive to specific climate conditions. Next challenge is to correlate the variation and adaptive response at the phenotypic, transcriptome and genome level with the micro-environment that these populations face. In addition, we have collected field temperature data every 60 – 90 minutes for seven years in the soil and the air at flowering height. These data need to be analyzed to look for

patterns and temperature variability in locations of our 16 populations. After gathering these detailed micro-climate temperature data, we will then explore the relationship between micro-climate temperature with avoidance and tolerance strategies and difference in gene expression as well as candidate genes for adapting to each elevation. Since temperature variation greatly affects plants' responses to stress, our results from the dissertation can be better understood when connected with the specific temperature that plants face in their environment.

In nature, plants actually experience several stressors at the same time. Combined stress response is not the direct addition of the two stresses (Rizhsky et al. 2002, Rizhsky et al. 2004b). Studying the combined effect of and adaptation to multiple stressors will be an important and productive future research topic. The ultimate goal is to transfer our knowledge on stress response into general knowledge into other species. By transferring the knowledge into crops, we can design plants that emphasize either tolerance or avoidance strategy in stress response while minimizing loss of yield or quality of the crops. By studying universal stresses, as important factors that affect species' ecological evolution and community composition, I hope my dissertation has contributed to the understanding of how species evolve under stress and how species will evolve under current and future stress.

Lastly, besides of the exciting results from studies in the dissertation, this dissertation also generated large amount of data, especially from the transcriptome and genome studies. Our findings in this dissertation initiated a good start in exploring these data, but we barely even touched the surface of these data. These data are important treasures from my dissertation and will play an important role in our understanding of many other key questions, generating collaborations and producing very inspiring results as we dive deeply in the data. I am very excited to see the data being used in many different ways. We will be willing to share these data

with any potential collaborators. This is just a door to a completely new and exciting world for our understanding on stress response!

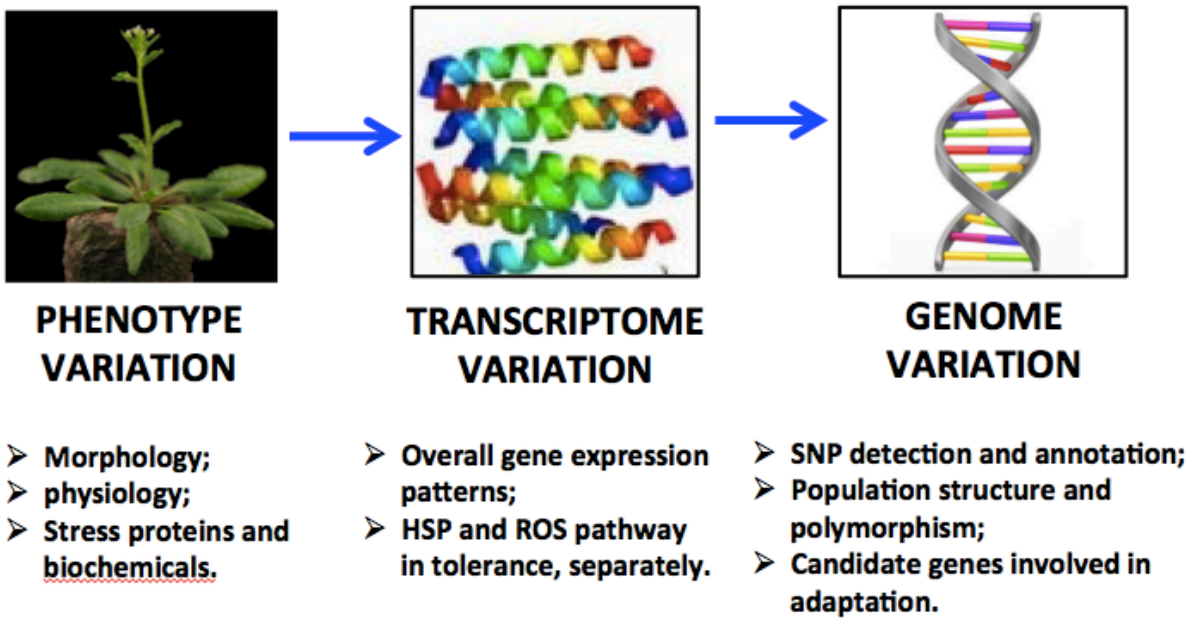


Figure 29. A framework for studying stress response. Under each level, we performed the measures or analysis below.

APPENDIX A

SUPPLEMENTARY MATERIALS FOR CHAPTER TWO

Table 14. Population locations and climate principle components.

Elevation (m)	Population	Longitude	Latitude	Climate PC1	Climate PC2
109	PIN	2.6591	41.6592	-3.6458	-1.7597
110	RAB	3.0537	42.3781	-4.4803	0.8848
332	SPE	2.9162	41.9275	-2.9213	-1.6334
340	BAR	2.1278	41.4322	-4.58	-1.1973
351	HOR	2.6202	41.6645	-2.7083	-1.8691
440	ARB	2.4941	41.8143	-2.1884	-0.6771
519	COC	3.1653	42.3263	-3.2761	-0.087
597	POB	1.0235	41.3526	-2.0028	3.7342
836	MUR	2.0002	41.6757	-2.0033	0.2208
912	VDM	1.0249	42.0292	1.2933	2.6184
1163	ALE	1.3187	42.4105	2.3143	1.2497
1397	BIS	0.5334	42.4878	5.8196	-2.246
1491	PAL	1.2926	42.4312	4.2579	0.6184
1538	VIE	0.7606	42.6256	5.7257	-1.1941
1664	PAN	-0.2310	42.7620	5.7899	-1.2883

Table 15. Constitutive SA concentrations from 2009 Experiment with eight populations.

Elevation(m)	Population	Family	Rpp	SAF(ug/g wet mass)	SAT(ug/g wet mass)	LnSAF	LnSAT	AveLnSAF	AveLnSAT
340	BAR	2	1	5.87	16.41	1.77	2.80	1.77	2.76
340	BAR	2	2	4.87	13.54	1.58	2.61		
340	BAR	3	1	3.73	11.98	1.32	2.48		
340	BAR	3	2	7.57	20.25	2.02	3.01		
340	BAR	6	1	9.85	30.56	2.29	3.42		
340	BAR	6	2	11.85	34.71	2.47	3.55		
340	BAR	8	1	5.96	16.48	1.78	2.80		
340	BAR	8	2	5.42	14.06	1.69	2.64		
340	BAR	10	1	6.05	16.38	1.80	2.80		
340	BAR	10	2	5.22	14.89	1.65	2.70		
340	BAR	12	1	5.92	15.38	1.78	2.73		
340	BAR	12	2	5.51	11.53	1.71	2.46		
340	BAR	14	1	5.84	16.81	1.77	2.82		
340	BAR	14	2	4.38	11.78	1.48	2.47		
340	BAR	15	1	6.89	17.73	1.93	2.88		
340	BAR	15	2	5.26	8.50	1.66	2.14		
340	BAR	16	1	4.92	14.65	1.59	2.68		
340	BAR	16	2	4.74	15.14	1.56	2.72		
351	HOR	1	1	6.60	20.55	1.89	3.02	1.69	2.69
351	HOR	1	2	5.53	15.66	1.71	2.75		
351	HOR	2	1	6.91	22.41	1.93	3.11		
351	HOR	2	2	7.91	29.95	2.07	3.40		
351	HOR	3	1	4.77	13.29	1.56	2.59		
351	HOR	3	2	1.72	9.68	0.54	2.27		
351	HOR	5	1	5.22	12.04	1.65	2.49		
351	HOR	5	2	6.21	10.63	1.83	2.36		
351	HOR	9	1	5.44	11.99	1.69	2.48		
351	HOR	9	2	4.35	10.75	1.47	2.38		
351	HOR	12	1	4.43	9.93	1.49	2.30		
351	HOR	12	2	5.83	14.79	1.76	2.69		
351	HOR	13	1	5.41	13.24	1.69	2.58		
351	HOR	13	2	5.57	12.20	1.72	2.50		
351	HOR	19	1	6.04	19.93	1.80	2.99		
351	HOR	19	2	9.96	26.79	2.30	3.29		
351	HOR	20	1	4.72	14.23	1.55	2.66		
351	HOR	20	2	5.55	13.86	1.71	2.63		
440	ARB	5	1	4.32	10.64	1.46	2.36	1.43	2.50
440	ARB	9	1	2.98	8.36	1.09	2.12		
440	ARB	12	1	4.60	17.44	1.53	2.86		
440	ARB	15	1	4.75	11.67	1.56	2.46		
440	ARB	17	1	4.60	10.04	1.53	2.31		
440	ARB	19	1	5.44	14.89	1.69	2.70		
440	ARB	23	1	6.74	16.44	1.91	2.80		
440	ARB	25	1	2.35	15.32	0.86	2.73		
440	ARB	26	1	3.49	6.82	1.25	2.18		
597	POB	2	1	5.60	14.97	1.69	2.71	1.47	2.60
597	POB	6	1	3.72	9.26	1.31	2.23		
597	POB	7	1	6.00	15.25	1.79	2.72		
597	POB	9	1	5.36	18.58	1.68	2.92		
597	POB	13	1	3.79	10.21	1.33	2.32		
597	POB	17	1	3.03	10.53	1.11	2.35		
597	POB	18	1	4.94	16.15	1.60	2.78		
597	POB	19	1	3.98	14.59	1.38	2.68		
597	POB	20	1	3.91	15.18	1.36	2.72		
836	MUR	1	1	3.07	6.66	1.12	2.16	1.39	2.46
836	MUR	2	1	3.66	8.36	1.30	2.10		
836	MUR	7	1	3.42	9.88	1.23	2.29		
836	MUR	8	1	2.95	8.96	1.08	2.19		
836	MUR	12	1	5.12	17.29	1.63	2.85		
836	MUR	13	1	3.17	8.36	1.15	2.12		
836	MUR	17	1	4.94	15.88	1.60	2.77		
836	MUR	18	1	6.20	19.63	1.83	2.98		
836	MUR	21	1	4.64	14.16	1.54	2.65		
912	VDM	1	1	5.61	20.23	1.72	3.01	1.36	2.58
912	VDM	2	1	3.45	11.04	1.24	2.40		
912	VDM	3	1	2.81	13.13	1.03	2.58		
912	VDM	5	1	5.04	15.75	1.62	2.76		
912	VDM	6	1	4.23	17.81	1.44	2.88		
912	VDM	8	1	3.30	14.63	1.19	2.70		
912	VDM	13	1	3.51	11.00	1.25	2.40		
912	VDM	14	1	4.23	9.95	1.44	2.30		
912	VDM	17	1	3.69	9.26	1.30	2.23		
1163	ALE	3	1	3.69	13.13	1.31	2.57	1.30	2.36
1163	ALE	3	2	4.47	12.97	1.50	2.56		
1163	ALE	4	1	3.97	10.34	1.38	2.34		
1163	ALE	4	2	4.70	11.83	1.55	2.47		
1163	ALE	5	1	3.90	9.48	1.36	2.25		
1163	ALE	5	2	4.82	10.81	1.57	2.38		
1163	ALE	8	1	5.02	10.95	1.61	2.39		
1163	ALE	8	2	3.23	9.15	1.17	2.21		
1163	ALE	9	1	4.51	11.88	1.51	2.47		
1163	ALE	9	2	4.01	8.76	1.39	2.17		
1163	ALE	12	1	4.67	13.31	1.54	2.59		
1163	ALE	12	2	3.94	9.83	1.37	2.29		
1163	ALE	13	1	3.90	11.43	1.36	2.44		
1163	ALE	13	2	4.20	9.68	1.43	2.27		
1163	ALE	17	1	4.25	10.32	1.45	2.33		
1163	ALE	17	2	5.01	11.13	1.61	2.41		
1163	ALE	20	1	3.21	7.69	1.17	2.04		
1163	ALE	20	2	0.44	10.67	-0.82	2.37		
1538	VIE	1	1	4.90	10.79	1.59	2.38	1.48	2.31
1538	VIE	1	2	3.95	10.36	1.37	2.34		
1538	VIE	3	1	5.74	14.70	1.75	2.69		
1538	VIE	3	2	4.14	8.73	1.42	2.17		
1538	VIE	4	1	5.13	11.43	1.64	2.44		
1538	VIE	4	2	3.72	7.01	1.31	1.95		
1538	VIE	9	1	6.11	13.02	1.81	2.57		
1538	VIE	9	2	3.88	8.11	1.36	2.09		
1538	VIE	11	1	5.40	14.70	1.69	2.69		
1538	VIE	11	2	3.77	9.95	1.33	2.30		
1538	VIE	12	1	4.49	14.70	1.50	2.69		
1538	VIE	12	2	4.00	7.76	1.39	2.05		
1538	VIE	18	1	3.21	7.80	1.17	2.05		
1538	VIE	18	2	3.92	10.55	1.37	2.36		
1538	VIE	19	1	4.49	8.24	1.50	2.11		
1538	VIE	19	2	3.92	8.48	1.37	2.14		
1538	VIE	20	1	4.43	9.49	1.49	2.25		
1538	VIE	20	2	4.79	10.04	1.57	2.31		

Table 16. Constitutive SA concentrations from 2013 Experiment with 15 populations.

Elevation	Population	Family	SAF(ug/g dry mass)	SAT(ug/g dry mass)	LnSAF	LnSAT	AveLnSAF	AveLnSAT
109	PIN	3	17.5	25.6	2.86	3.24	2.88	3.34
109	PIN	6	8.8	17.3	2.18	2.85	.	.
109	PIN	7	34.0	49.5	3.53	3.90	.	.
109	PIN	9	18.9	28.8	2.94	3.36	.	.
110	RAB	4	63.8	91.1	4.16	4.51	3.40	3.73
110	RAB	9	30.6	34.7	3.42	3.55	.	.
110	RAB	17	22.8	32.0	3.13	3.47	.	.
110	RAB	20	17.9	29.2	2.88	3.37	.	.
332	SPE	2	13.7	19.9	2.62	2.99	3.15	3.50
332	SPE	5	15.5	20.1	2.74	3.00	.	.
332	SPE	6	102.3	161.1	4.63	5.08	.	.
332	SPE	7	13.7	18.4	2.62	2.91	.	.
340	BAR	3	8.8	8.6	2.17	2.15	2.99	2.87
340	BAR	4	19.5	21.7	2.97	3.08	.	.
340	BAR	9	24.9	29.4	3.22	3.38	.	.
340	BAR	11	35.8	.	3.58	.	.	.
351	HOR	4	58.6	55.9	4.07	4.02	3.79	3.87
351	HOR	6	34.0	46.4	3.53	3.84	.	.
351	HOR	7	37.3	42.7	3.62	3.75	.	.
351	HOR	16	52.2	.	3.95	.	.	.
440	ARB	3	6.7	6.1	1.90	1.81	2.59	2.72
440	ARB	6	20.8	24.6	3.03	3.20	.	.
440	ARB	8	10.9	12.5	2.39	2.52	.	.
440	ARB	10	20.5	28.4	3.02	3.35	.	.
519	COC	7	41.1	52.6	3.72	3.96	3.57	3.65
519	COC	14	14.0	27.3	2.64	3.31	.	.
519	COC	17	127.0	101.9	4.84	4.62	.	.
519	COC	19	22.2	15.2	3.10	2.72	.	.
597	POB	7	9.1	15.7	2.21	2.75	2.79	3.34
597	POB	10	57.4	67.0	4.05	4.21	.	.
597	POB	16	11.9	32.4	2.48	3.48	.	.
597	POB	19	11.4	18.9	2.44	2.94	.	.
836	MUR	15	14.0	27.4	2.64	3.31	2.26	2.89
836	MUR	16	10.0	14.8	2.30	2.70	.	.
836	MUR	17	10.0	20.1	2.31	3.00	.	.
836	MUR	20	5.9	12.6	1.78	2.53	.	.
912	VDM	9	29.0	35.7	3.37	3.57	2.89	3.38
912	VDM	11	18.6	33.7	2.92	3.52	.	.
912	VDM	17	16.6	29.1	2.81	3.37	.	.
912	VDM	20	11.5	21.6	2.45	3.07	.	.
1163	ALE	4	20.0	14.8	3.00	2.70	2.70	2.84
1163	ALE	10	13.4	22.2	2.60	3.10	.	.
1163	ALE	12	12.2	15.4	2.51	2.73	.	.
1163	ALE	16	.	.	.	.	.	.
1397	BIS	8	25.7	35.1	3.25	3.56	3.04	3.44
1397	BIS	11	20.5	36.6	3.02	3.60	.	.
1397	BIS	16	17.8	30.7	2.88	3.42	.	.
1397	BIS	20	20.1	24.2	3.00	3.19	.	.
1491	PAL	6	3.8	18.4	1.33	2.91	2.05	3.16
1491	PAL	7	18.0	29.4	2.89	3.38	.	.
1491	PAL	12	6.8	24.1	1.92	3.18	.	.
1491	PAL	16	.	.	.	.	.	.
1538	VIE	3	13.7	19.5	2.62	2.97	2.83	3.00
1538	VIE	4	21.2	18.2	3.05	2.90	.	.
1538	VIE	6	17.6	25.5	2.87	3.24	.	.
1538	VIE	16	16.0	17.9	2.77	2.89	.	.
1664	PAN	1	4.9	10.7	1.59	2.37	1.89	2.44
1664	PAN	5	5.7	13.8	1.74	2.63	.	.
1664	PAN	8	5.4	11.1	1.69	2.41	.	.
1664	PAN	9	12.9	10.4	2.55	2.34	.	.

Table 17. Regression analysis for leaf free salicylic acid concentration as a function of a) climate PC2 alone and b) combination of climate PC1 and PC2.

Leaf Free SA Concentration as a function of:

ClimatePC1

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	1	1.082	1.0817	5.05	0.043
ClimatePC1	1	1.082	1.0817	5.05	0.043
Error	13	2.787	0.2143		
Total	14	3.868			

S = 0.462977 R-sq = 27.96%

ClimatePC2

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	1	0.08942	0.08942	0.31	0.589
ClimatePC2	1	0.08942	0.08942	0.31	0.589
Error	13	3.77883	0.29068		
Total	14	3.86824			

S = 0.539147 R-sq = 2.31%

ClimatePC1 and ClimatePC2

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	2	1.2253	0.6127	2.78	0.102
ClimatePC1	1	1.1359	1.1359	5.16	0.042
ClimatePC2	1	0.1436	0.1436	0.65	0.435
Error	12	2.6429	0.2202		
Total	14	3.8682			

S = 0.469302 R-sq = 31.68%

Table 18. Constitutive and heat treatment induced SA concentrations for the four focal populations (BAR, HOR, ALE, and VIE) in the 2009 Experiment.

				Control (Ave +/- SE)		Heat Treatment (Ave +/- SE)	
Elev	Pop	Fam	N	Free SA (ug/g wet mass)	Total SA (ug/g wet mass)	Free SA (ug/g wet mass)	Total SA (ug/g wet mass)
340	BAR	2	2	5.37 +/- 0.50	14.98 +/- 1.43	4.07 +/- 0.59	10.24 +/- 1.45
340	BAR	3	2	5.65 +/- 1.92	16.11 +/- 4.13	4.26 +/- 1.24	14.20 +/- 3.04
340	BAR	6	2	10.85 +/- 1.00	32.63 +/- 2.07	7.34 +/- 1.66	20.23 +/- 3.63
340	BAR	8	2	5.69 +/- 0.27	15.27 +/- 1.21	5.33 +/- 0.75	14.59 +/- 0.23
340	BAR	10	2	5.64 +/- 0.41	15.63 +/- 0.75	6.82 +/- 0.56	14.52 +/- 1.51
340	BAR	12	2	5.72 +/- 0.20	13.46 +/- 1.93	6.18 +/- 0.83	14.75 +/- 0.63
340	BAR	14	2	5.11 +/- 0.73	14.29 +/- 2.51	5.29 +/- 1.34	14.03 +/- 5.20
340	BAR	15	2	6.07 +/- 0.82	13.11 +/- 4.62	5.12 +/- 0.45	13.18 +/- 0.34
340	BAR	16	2	4.83 +/- 0.09	14.90 +/- 0.24	4.82 +/- 0.28	10.87 +/- 0.95
351	HOR	1	2	6.07 +/- 0.54	18.10 +/- 2.45	6.02 +/- 0.06	19.17 +/- 1.38
351	HOR	2	2	7.41 +/- 0.50	26.18 +/- 3.77	7.29 +/- 0.76	18.48 +/- 1.53
351	HOR	3	2	3.25 +/- 1.52	11.49 +/- 1.81	4.73 +/- 0.82	12.19 +/- 3.44
351	HOR	5	2	5.71 +/- 0.49	11.33 +/- 0.70	4.45 +/- 0.07	10.92 +/- 0.24
351	HOR	9	2	4.89 +/- 0.55	11.37 +/- 0.62	4.76 +/- 0.80	12.09 +/- 0.77
351	HOR	12	2	5.13 +/- 0.70	12.36 +/- 2.43	4.36 +/- 0.63	10.71 +/- 1.87
351	HOR	13	2	5.49 +/- 0.08	12.72 +/- 0.52	4.65 +/- 0.60	12.46 +/- 2.14
351	HOR	19	2	8.00 +/- 1.96	23.36 +/- 3.43	7.30 +/- 0.33	26.07 +/- 3.76
351	HOR	20	2	5.13 +/- 0.42	14.04 +/- 0.18	4.56 +/- 0.37	13.53 +/- 3.27
1163	ALE	3	2	4.08 +/- 0.39	13.05 +/- 0.08	4.34 +/- 0.25	12.47 +/- 2.20
1163	ALE	4	2	4.33 +/- 0.36	11.09 +/- 0.74	4.47 +/- 0.81	11.73 +/- 1.48
1163	ALE	5	2	4.36 +/- 0.46	10.15 +/- 0.66	2.82 +/- 0.50	7.56 +/- 0.02
1163	ALE	8	2	4.13 +/- 0.89	10.05 +/- 0.90	3.78 +/- 0.27	8.40 +/- 0.96
1163	ALE	9	2	4.26 +/- 0.25	10.32 +/- 1.56	3.65 +/- 0.21	8.15 +/- 0.31
1163	ALE	12	2	4.30 +/- 0.37	11.57 +/- 1.74	4.25 +/- 0.00	9.47 +/- 0.32
1163	ALE	13	2	4.05 +/- 0.15	10.56 +/- 0.87	4.21 +/- 0.97	9.33 +/- 2.54
1163	ALE	17	2	4.63 +/- 0.38	10.72 +/- 0.41	4.83 +/- 0.73	11.72 +/- 2.24
1163	ALE	20	2	3.21 +/- NA	9.18 +/- 1.49	2.52 +/- 0.75	10.61 +/- 3.99
1538	VIE	1	2	4.43 +/- 0.48	10.58 +/- 0.21	3.73 +/- 0.39	10.34 +/- 1.85
1538	VIE	3	2	4.94 +/- 0.80	11.72 +/- 2.99	3.01 +/- 0.83	7.31 +/- 2.29
1538	VIE	4	2	4.43 +/- 0.71	9.22 +/- 2.21	4.23 +/- 0.52	9.52 +/- 1.08
1538	VIE	9	2	5.00 +/- 1.11	10.57 +/- 2.45	4.33 +/- 1.07	6.41 +/- NA
1538	VIE	11	2	4.59 +/- 0.81	12.33 +/- 2.37	3.87 +/- 0.35	10.51 +/- 0.07
1538	VIE	12	2	4.24 +/- 0.24	11.24 +/- 3.46	2.62 +/- 0.61	8.28 +/- 0.95
1538	VIE	18	2	3.57 +/- 0.35	9.18 +/- 1.38	3.63 +/- 0.09	7.57 +/- 1.79
1538	VIE	19	2	4.20 +/- 0.28	8.36 +/- 0.12	3.45 +/- 0.52	7.76 +/- 0.28
1538	VIE	20	2	4.61 +/- 0.18	9.76 +/- 0.28	3.26 +/- 0.25	8.15 +/- 0.76

Table 19. Camalexin concentrations for 11 populations in the 2013 Experiment**.**

Elevation	Population	Family	Camalexin (ug/g dry mass)	LnCamalexin	AveLnCamalexin
332	SPE	2	50.5	3.92	4.98 +/- 0.78
332	SPE	5	0.0	NA	
332	SPE	6	674.5	6.51	
332	SPE	7	90.4	4.50	
440	ARB	3	0.0	NA	4.16 +/- 0.65
440	ARB	6	204.6	5.32	
440	ARB	8	20.9	3.04	
440	ARB	10	61.7	4.12	
519	COC	7	7.2	1.98	2.25 +/- 0.13
519	COC	14	0.0	NA	
519	COC	17	10.6	2.36	
519	COC	19	11.2	2.42	
597	POB	7	3.7	1.32	2.85 +/- 1.09
597	POB	10	169.7	5.13	
597	POB	16	74.0	4.30	
597	POB	19	1.9	0.66	
836	MUR	15	74.1	4.30	2.75 +/- 0.58
836	MUR	16	4.4	1.47	
836	MUR	17	13.2	2.58	
836	MUR	20	14.3	2.66	
912	VDM	9	0.0	NA	2.93 +/- 1.10
912	VDM	11	136.4	4.92	
912	VDM	17	3.0	1.10	
912	VDM	20	16.4	2.80	
1163	ALE	4	34.8	3.55	1.67 +/- 1.01
1163	ALE	10	1.1	0.07	
1163	ALE	12	4.0	1.40	
1163	ALE	16	NA	NA	
1397	BIS	8	1.2	0.18	1.21 +/- 0.41
1397	BIS	11	3.3	1.18	
1397	BIS	16	9.2	2.22	
1397	BIS	20	3.5	1.26	
1491	PAL	6	17.5	2.86	2.75 +/- 0.06
1491	PAL	7	15.9	2.76	
1491	PAL	12	14.0	2.64	
1491	PAL	16	NA	NA	
1538	VIE	3	9.6	2.26	2.77 +/- 0.86
1538	VIE	4	0.0	NA	
1538	VIE	6	5.0	1.60	
1538	VIE	16	87.5	4.47	
1664	PAN	1	0.7	-0.34	0.19 +/- 0.30
1664	PAN	5	1.3	0.23	
1664	PAN	8	2.0	0.71	
1664	PAN	9	0.0	NA	

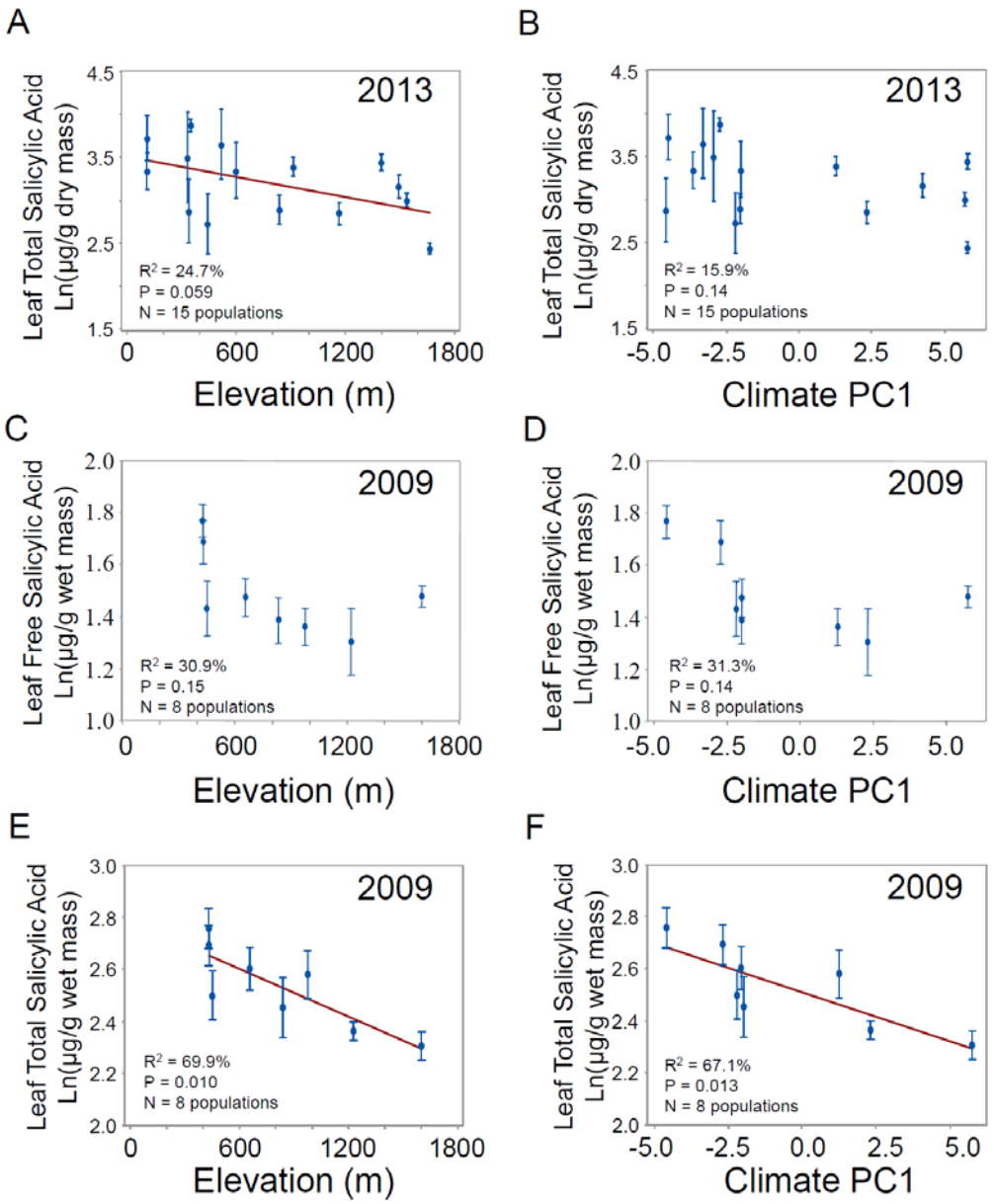


Figure 30. Scatterplots showing relationships between leaf total salicylic acid concentration and A) source population elevation and B) climate PC1 in the 2013 experiment. Leaf free salicylic acid concentration as a function of C) source population elevation and D) climate PC1 in the 2009 experiment. Leaf total salicylic acid concentration as a function of E) source population elevation and F) climate PC1 in the 2009 experiment. Increasing values of climate PC1 indicate colder temperatures and greater rainfall. Shown are population means (+/- 1SE) for ten-week-old plants representing four maternal families per population in the 2013 experiment and four-week-old plants representing nine maternal families per population in the 2009 experiment.

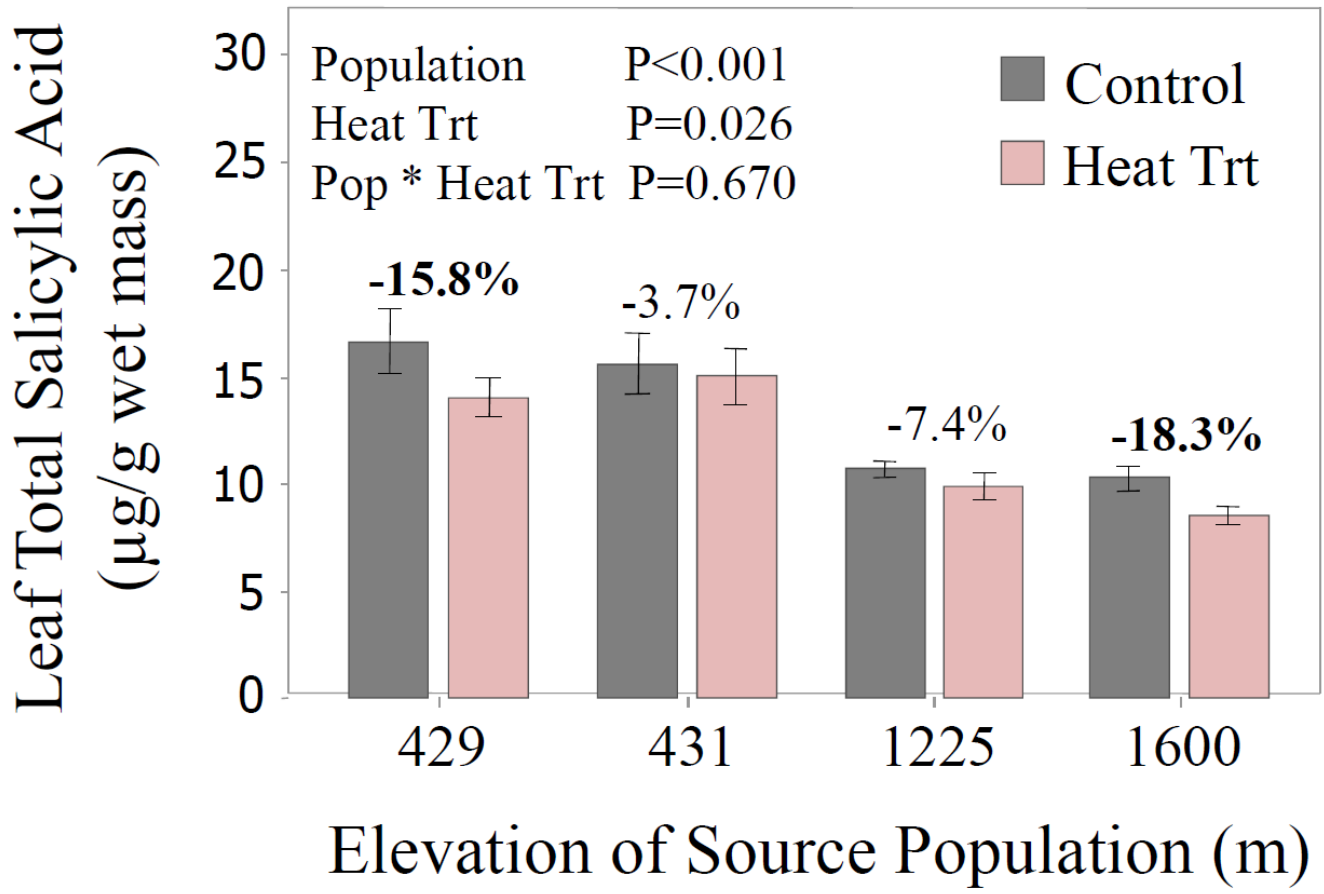


Figure 31. Leaf total salicylic acid concentrations for plants that received a heat treatment of 44ºC for 3 hr, relative to control plants that received the normal growth temperature of 20 ºC as part of the 2009 common garden experiment. Nine maternal lines were grown from each of the four source populations, BAR, HOR, ALE, and VIE, representing elevations of 429, 431, 1225, and 1600m, respectively. Four plants were grown from each maternal line and half were randomly assigned to receive the heat treatment and half to receive the normal growth temperature, for a total of 144 plants (Supplemental Table 5). Leaf tissues were collected three days after the conclusion of the heat treatment.

APPENDIX B

SUPPLMENTARY MATERIALS FOR CHAPTER THREE

Table 20. Means and standard deviations for the concentrations (ug/g dry mass) of camalexin, total SA, and free SA in control plants (C), cold-treated plants (I), and the induction response to cold (I - C).

		Control Plants at 20°C		Cold-Treated Plants at 10°C		Induction Response to Cold	
Trait	**Pop**	**N**	**Mean +/- SD**	**N**	**Mean +/- SD**	**N**	**Mean +/- SD**
Camalexin (ug/g)	ALE	8	3.3 +/- 7.4	9	4.9 +/- 9.5	8	1.2 +/- 4.2
	BAR	8	0.0 +/- 0.0	8	3.2 +/- 6.1	8	3.2 +/- 6.1
	HOR	8	1.1 +/- 3.0	8	24.7 +/- 70.0	8	23.7 +/- 70.5
	VIE	9	106.2 +/- 94.8	9	60.4 +/- 40.3	9	-45.8 +/-102.6
Total SA (ug/g)	ALE	8	16.2 +/- 10.1	9	37.7 +/- 35.8	8	23.7 +/- 37.3
	BAR	8	26.6 +/- 25.4	8	25.1 +/- 10.4	8	-1.5 +/- 26.5
	HOR	8	26.8 +/- 15.8	8	24.8 +/- 25.8	8	-2.0 +/- 33.5
	VIE	9	34.3 +/- 26.6	9	37.3 +/- 15.5	9	3.1 +/- 30.4
Free SA (ug/g)	ALE	8	8.5 +/- 3.3	9	21.0 +/- 21.5	8	6.0 +/- 9.3
	BAR	8	10.6 +/- 6.7	8	13.9 +/- 6.2	8	3.3 +/- 9.2
	HOR	8	10.8 +/- 3.3	8	14.0 +/- 10.0	8	3.2 +/- 7.7
	VIE	9	20.7 +/- 14.6	9	19.8 +/- 8.1	9	-0.9 +/- 15.9

Table 21. Means and standard deviations for leaf bacterial titers and symptoms in the structured populations.

		Leaf Bacterial Titer (Log(cfu/disk))		Leaf Disease (% symptoms)	
Population	**Genotype**	**N**	**Mean +/- SD**	**N**	**Mean +/- SD**
ALE	3	2	6.0 +/- 0.4	4	90 +/- 20.0
	4	1	6.3 +/- NA	4	100 +/- 0.0
	5	2	5.2 +/- 1.7	4	100 +/- 0.0
	8	2	7.0 +/- 0.9	4	100 +/- 0.0
	9	2	5.8 +/- 0.1	4	100 +/- 0.0
	12	2	6.6 +/- 0.6	4	100 +/- 0.0
	13	2	6.5 +/- 0.0	4	100 +/- 0.0
	17	2	6.0 +/- 1.1	4	100 +/- 0.0
	20	2	7.2 +/- 0.3	4	100 +/- 0.0
BAR	2	2	5.6 +/- 1.6	4	85 +/- 30.0
	3	2	5.4 +/- 0.8	4	90 +/- 11.5
	6	2	6.5 +/- 0.5	4	100 +/- 0.0
	8	2	5.8 +/- 0.5	4	95 +/- 10.0
	10	2	6.7 +/- 0.7	4	100 +/- 0.0
	12	2	6.3 +/- 1.0	2	100 +/- 0.0
	14	2	6.7 +/- 0.1	4	95 +/- 10.0
	15	2	6.6 +/- 0.5	4	85 +/- 19.1
	16	2	6.0 +/- 1.0	4	100 +/- 0.0
HOR	1	2	6.0 +/- 0.9	4	95 +/- 10.0
	2	1	7.5 +/- NA	4	95 +/- 10.0
	3	2	6.6 +/- 0.4	4	100 +/- 0.0
	5	2	5.3 +/- 1.3	4	100 +/- 0.0
	9	2	7.0 +/- 0.3	4	85 +/- 19.1
	12	2	5.9 +/- 0.8	4	100 +/- 0.0
	13	2	6.3 +/- 1.6	4	95 +/- 10.0
	19	2	6.4 +/- 0.0	4	100 +/- 0.0
	20	2	7.1 +/- 0.1	2	100 +/- 0.0
VIE	1	2	5.4 +/- 1.1	4	100 +/- 0.0
	3	2	4.8 +/- 0.5	4	70 +/- 34.6
	4	2	4.9 +/- 0.9	4	75 +/- 30.0
	9	2	6.0 +/- 0.3	4	85 +/- 30.0
	11	2	5.2 +/- 0.6	4	75 +/- 30.0
	12	2	5.0 +/- 0.5	4	90 +/- 20.0
	18	2	5.8 +/- 0.8	4	55 +/- 30.0
	19	2	4.9 +/- 1.2	4	90 +/- 20.0
	20	2	6.2 +/- 0.1	4	85 +/- 30.0

Table 22. Means and standard deviations for the concentrations (ug/g dry mass) of camalexin, total SA, and free SA in control plants (C), plants infected with Pst DC3000 (I), and the induction response to infection (I - C).

		Control Plants with Mock Inoculation				Plants Infected with *Pst* DC3000				Induction Response to Infection			
Pop	Genotype	N	SAF(ug/g)	SAT(ug/g)	CAM(ug/g)	N	SAF(ug/g)	SAT(ug/g)	CAM(ug/g)	N	SAF(ug/g)	SAT(ug/g)	CAM(ug/g)
ALE	3	2	54.2 +/- 10.0	72.3 +/- 5.1	0.0 +/- 0.0	1	135.8 +/- NA	198.5 +/- NA	59.1 +/- NA	1	74.6 +/- NA	129.9 +/- NA	59.1 +/- NA
	4	2	34.7 +/- 7.8	63.1 +/- 13.0	15.7 +/- 11.2	2	71 +/- 20.1	161.0 +/- 97.4	109.3 +/- 69.5	2	36.3 +/- 12.3	97.9 +/- 84.4	93.6 +/- 58.3
	5	2	29.7 +/- 14.5	60.3 +/- 43.0	10.2 +/- 14.4	2	76.9 +/- 43.2	188.4 +/- 104.7	71.6 +/- 8.5	2	47.3 +/- 28.7	128.1 +/- 61.7	61.4 +/- 22.9
	8	2	34.1 +/- 17.6	22.2 +/- 21.9	4.3 +/- 0.9	2	89.2 +/- 35.5	191.0 +/- 62.4	112.4 +/- 78.4	2	55.1 +/- 17.9	168.8 +/- 40.5	108.1 +/- 79.4
	9	2	27.5 +/- 13.7	52.9 +/- 19.0	8.5 +/- 9.2	2	72.9 +/- 59.2	148.4 +/- 103.1	62.9 +/- 46.4	2	45.4 +/- 73.0	95.5 +/- 122.0	54.5 +/- 37.2
	12	2	62.7 +/- 35.7	102.1 +/- 96.6	14.7 +/- 20.8	2	95.7 +/- 66.5	341.5 +/- 215.4	132.1 +/- 11.9	2	33 +/- 30.8	239.3 +/- 118.8	117.4 +/- 8.9
	13	2	26.3 +/- 24.2	63.3 +/- 70.3	43.8 +/- 62.0	1	67.6 +/- NA	201.6 +/- NA	87.0 +/- NA	1	24.2 +/- NA	88.6 +/- NA	-0.7 +/- NA
	17	2	37.1 +/- 1.2	55.6 +/- 2.7	10.2 +/- 14.4	1	42.8 +/- NA	118.8 +/- NA	71.1 +/- NA	1	4.9 +/- NA	61.3 +/- NA	50.7 +/- NA
	20	2	37.9 +/- 38.6	102.2 +/- 98.0	49.8 +/- 46.4	2	42.1 +/- 34.4	138.9 +/- 100.3	99.3 +/- 97.9	2	4.2 +/- 72.9	36.7 +/- 198.4	49.5 +/- 144.3
BAR	1	1	47.2 +/- NA	34.3 +/- NA	15.6 +/- NA	1	48.2 +/- NA	112.6 +/- NA	46.0 +/- NA	1	1 +/- NA	78.3 +/- NA	30.4 +/- NA
	2	1	14.1 +/- NA	31.4 +/- NA	2.4 +/- NA	2	130.5 +/- 99.2	278.2 +/- 80.3	124.6 +/- 44.2	1	186.6 +/- NA	303.5 +/- NA	91.0 +/- NA
	3	2	16.7 +/- 9.3	29 +/- 2.9	9.6 +/- 13.6	2	54.8 +/- 36.3	103.4 +/- 44.6	68.6 +/- 59.1	2	38.1 +/- 27.0	74.4 +/- 41.7	59.0 +/- 72.7
	8	2	19.2 +/- 2.4	44.6 +/- 1.9	16.3 +/- 20.8	2	97.5 +/- 85.7	160.8 +/- 88.4	59.6 +/- 15.9	2	78.3 +/- 83.3	116.2 +/- 90.4	43.4 +/- 36.7
	10	2	70.8 +/- 31.3	113 +/- 57.2	16.9 +/- 21.5	2	384.3 +/- 406.6	613.1 +/- 638.1	108.2 +/- 92.9	2	313.5 +/- 437.8	500.1 +/- 695.4	91.4 +/- 71.4
	14	2	38.8 +/- 4.5	103.8 +/- 10.3	27.9 +/- 30.0	2	62.6 +/- 31.1	145.6 +/- NA	51.6 +/- NA	2	23.8 +/- 26.5	-31.0 +/- 92.6	-2.1 +/- 6.5
	15	2	18.5 +/- 0.2	32.0 +/- 0.9	4.1 +/- 0.9	2	43.9 +/- 9.1	102.3 +/- 15.7	86.5 +/- 40.7	2	25.4 +/- 9.3	70.4 +/- 14.8	82.4 +/- 39.8
	18	2	43.0 +/- 29.7	70.8 +/- 20.5	2.2 +/- 1.3	2	123.1 +/- 69.6	281.7 +/- 127.0	123.7 +/- 38.0	2	80.1 +/- 39.9	210.9 +/- 106.5	121.5 +/- 36.7
HOR	1	2	28.4 +/- 13.0	68.1 +/- 15.3	37.2 +/- 52.7	1	76.2 +/- NA	243.9 +/- 75.4	96.1 +/- 35.2	2	9.7 +/- 40.8	175.8 +/- 90.7	58.9 +/- 17.4
	2	1	20.7 +/- NA	91.2 +/- NA	0.0 +/- NA	1	48.3 +/- NA	81.1 +/- NA	45.6 +/- NA	1	27.7 +/- NA	-10.1 +/- NA	45.6 +/- NA
	3	2	10.2 +/- 6.8	43.4 +/- 5.7	9.6 +/- 13.5	2	57 +/- 68.3	287.1 +/- 14.6	172.6 +/- 3.4	2	46.7 +/- 75.1	243.8 +/- 20.4	163.1 +/- 10.2
	5	2	57.9 +/- 5.8	131.2 +/- 25.1	20.3 +/- 3.1	2	68.1 +/- 8.5	197.3 +/- 35.1	115.4 +/- 47.8	2	10.1 +/- 2.7	66.0 +/- 10.0	95.2 +/- 50.9
	9	2	25.2 +/- 13.9	53.2 +/- 28.0	11.1 +/- 3.5	2	37.7 +/- 9.2	179.8 +/- 45.2	70.4 +/- 66.3	2	12.5 +/- 23.1	126.6 +/- 17.2	59.3 +/- 69.8
	12	2	29.8 +/- 12.6	64.7 +/- 28.7	34.8 +/- 46.3	1	217.4 +/- NA	493.8 +/- NA	321.0 +/- NA	1	178.7 +/- NA	408.9 +/- NA	253.4 +/- NA
	13	2	35.3 +/- 36.6	65.2 +/- 60.6	17.9 +/- 25.3	2	75.1 +/- 9.4	327.4 +/- 37.1	143.1 +/- 68.5	2	39.9 +/- 46.0	262.3 +/- 97.7	125.2 +/- 93.7
	19	1	57.7 +/- NA	94.5 +/- NA	0.0 +/- NA	2	36 +/- 11.6	68.1 +/- 11.0	57.5 +/- 8.5	1	-13.5 +/- NA	-18.6 +/- NA	51.5 +/- NA
VIE	1	1	79.6 +/- NA	241.4 +/- NA	210.9 +/- NA	1	76 +/- NA	260.1 +/- NA	75.4 +/- NA	0	NA +/- NA	NA +/- NA	NA +/- NA
	3	2	13.9 +/- 2.7	41.5 +/- 12.2	25.2 +/- 12.9	2	131.8 +/- 116.1	277.5 +/- 192.4	200.6 +/- 84.6	2	117.9 +/- 118.8	236.0 +/- 204.5	175.4 +/- 71.7
	4	2	43.4 +/- 13.5	69.2 +/- 22.3	68.7 +/- 21.6	2	169.5 +/- 117.2	388.4 +/- 251.5	133.5 +/- 43.1	2	126.1 +/- 103.6	319.2 +/- 229.2	64.7 +/- 21.5
	9	2	39.8 +/- 22.3	68.3 +/- 46.4	8.7 +/- 11.6	2	104 +/- 69.9	213.2 +/- 156.9	76 +/- 45.8	2	64.2 +/- 47.6	144.9 +/- 110.5	67.3 +/- 34.3
	11	2	18.7 +/- 23.1	52.9 +/- 19.5	18.5 +/- 0.1	2	63.4 +/- 31.0	221.4 +/- 101.0	111.2 +/- 58.2	2	44.7 +/- 54.1	168.6 +/- 120.6	92.7 +/- 58.1
	12	2	78.2 +/- 2.2	182.7 +/- 13.9	239.1 +/- 7.3	2	64.2 +/- 48.1	232.5 +/- 184.9	190.9 +/- 24.6	2	-14.0 +/- 46.0	49.8 +/- 198.7	-48.2 +/- 17.3
	18	2	55.0 +/- 7.9	99.5 +/- 6.0	44.5 +/- 30.8	2	67.6 +/- 48.8	199.6 +/- 178.8	178.3 +/- 75.9	2	12.6 +/- 56.7	100.1 +/- 184.7	133.8 +/- 106.7
	19	2	89.2 +/- 32.6	176.4 +/- 16.6	148.3 +/- 2.2	2	75.3 +/- 5.9	220.5 +/- 73.4	284.5 +/- 30.9	2	-13.9 +/- 38.5	44.1 +/- 56.8	136.2 +/- 28.7
	20	1	30.8 +/- NA	75.0 +/- NA	3.08 +/- NA	2	92.1 +/- 37.4	247.5 +/- 14.8	289.5 +/- 113.1	1	87.6 +/- NA	183.0 +/- NA	171.0 +/- NA

Table 23. Allele calls at the ACD6 locus (At4g14400) for each genotype.

			Allele Identified at the ACD6 Locus		
Elevation	**Population**	**Genotype**	**Est-1 Allele**	**KZ Allele**	**Col-0 Allele**
340	BAR	1 †	n.d.	n.d.	n.d.
340	BAR	2		X	
340	BAR	3	X		
340	BAR	8		X	
340	BAR	10		X	
340	BAR	14		X	
340	BAR	15	X		
340	BAR	18 †	n.d.	n.d.	n.d.
351	HOR	1	X		
351	HOR	2	X		
351	HOR	3		X	
351	HOR	5		X	
351	HOR	9		X	
351	HOR	12		X	
351	HOR	13		X	
351	HOR	19	X		
1163	ALE	3		X	
1163	ALE	4		X	
1163	ALE	5		X	
1163	ALE	8		X	
1163	ALE	9		X	
1163	ALE	12		X	
1163	ALE	13		X	
1163	ALE	17		X	
1163	ALE	20 *	n.d.	n.d.	n.d.
1538	VIE	1			X
1538	VIE	3			X
1538	VIE	4		X	X
1538	VIE	9		X	
1538	VIE	11			X
1538	VIE	12			X
1538	VIE	18			X
1538	VIE	19			X
1538	VIE	20			X

† No DNA collected. * No amplification achieved.

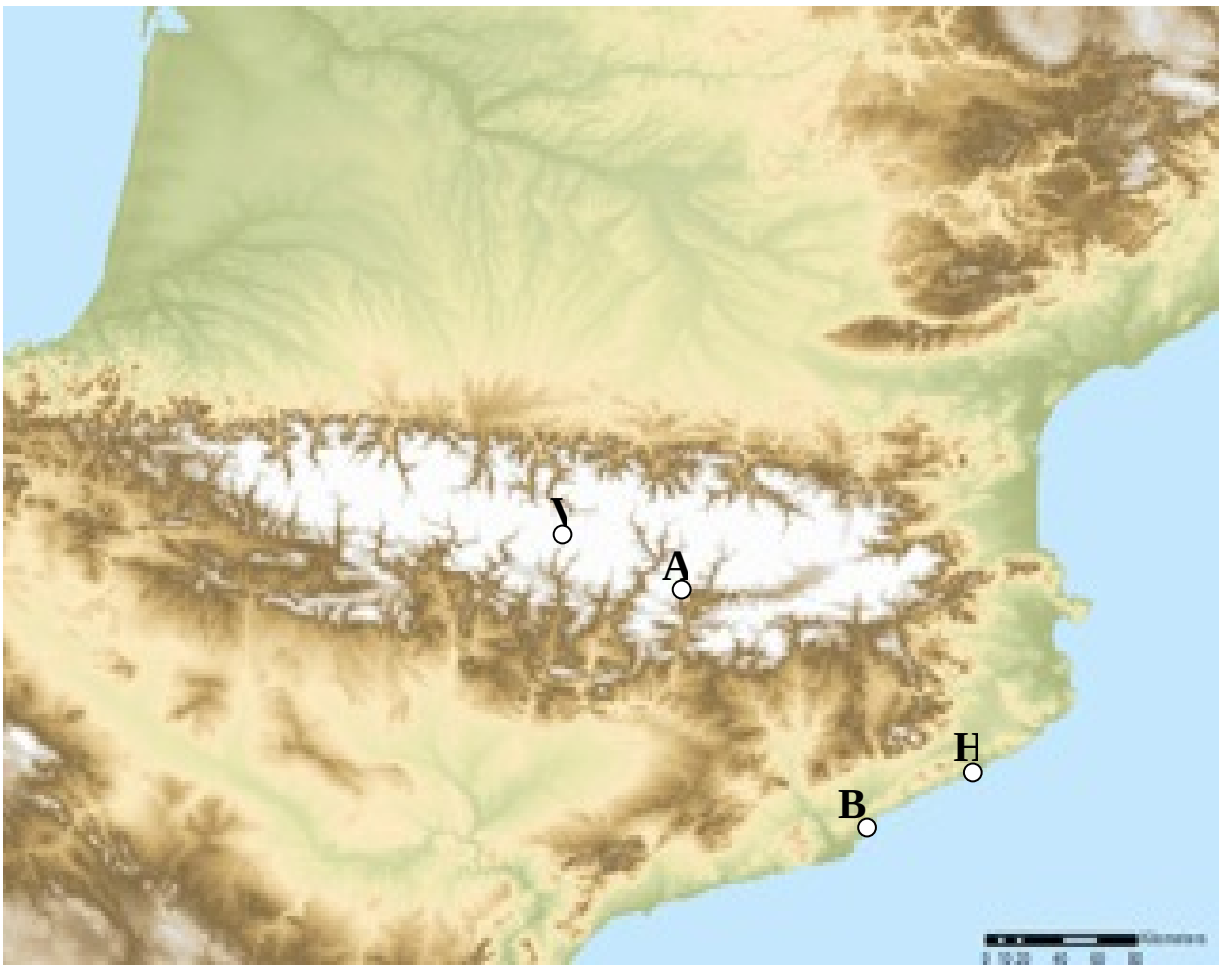


Figure 32. Locations of the four populations on the Iberian Peninsula.

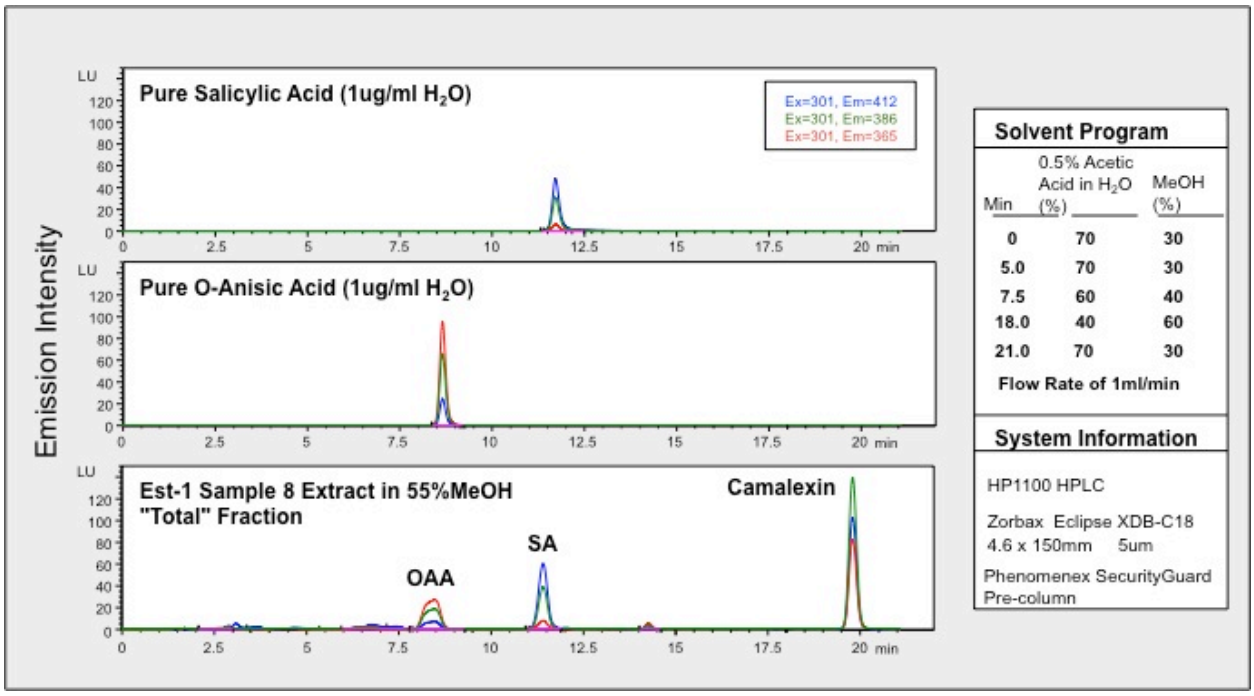


Figure 33. SA standard, OAA standard and Est-1 sample in HPLC system.

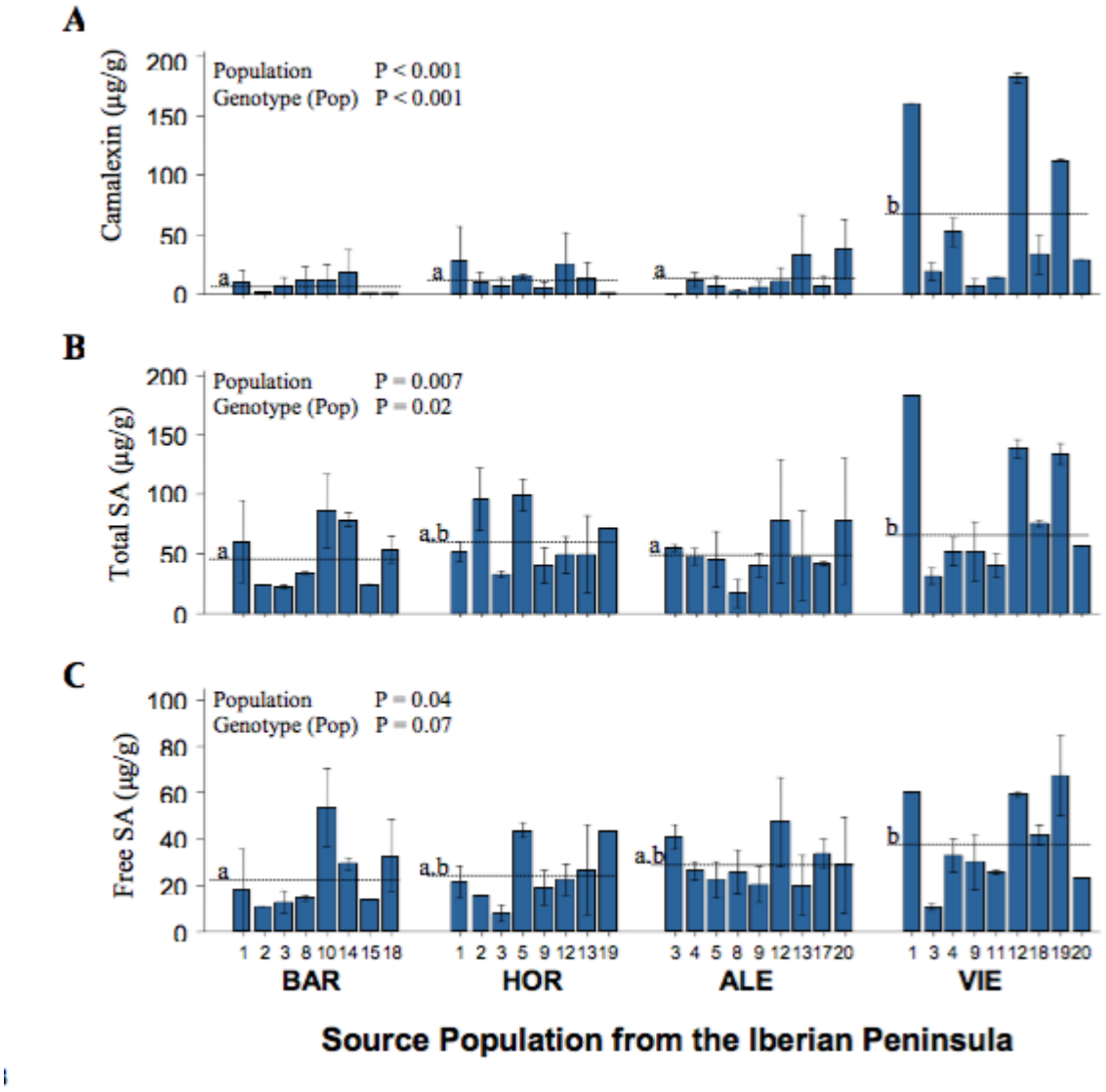


Figure 34. Constitutive genotype means for leaf concentration (µg/g dry weight) of A) camalexin, B) total salicylic acid, and C) free salicylic acid of plants grown under common garden conditions from four populations of *A. thaliana* on the Iberian Peninsula. Plants were mock inoculated with buffer solution as negative controls for the plants infected with bacteria. Shown are means (+/- 1SE) of two individuals of each genotype in each population. Dotted lines indicate population means. Letters indicate a significant difference among populations at P=0.05.

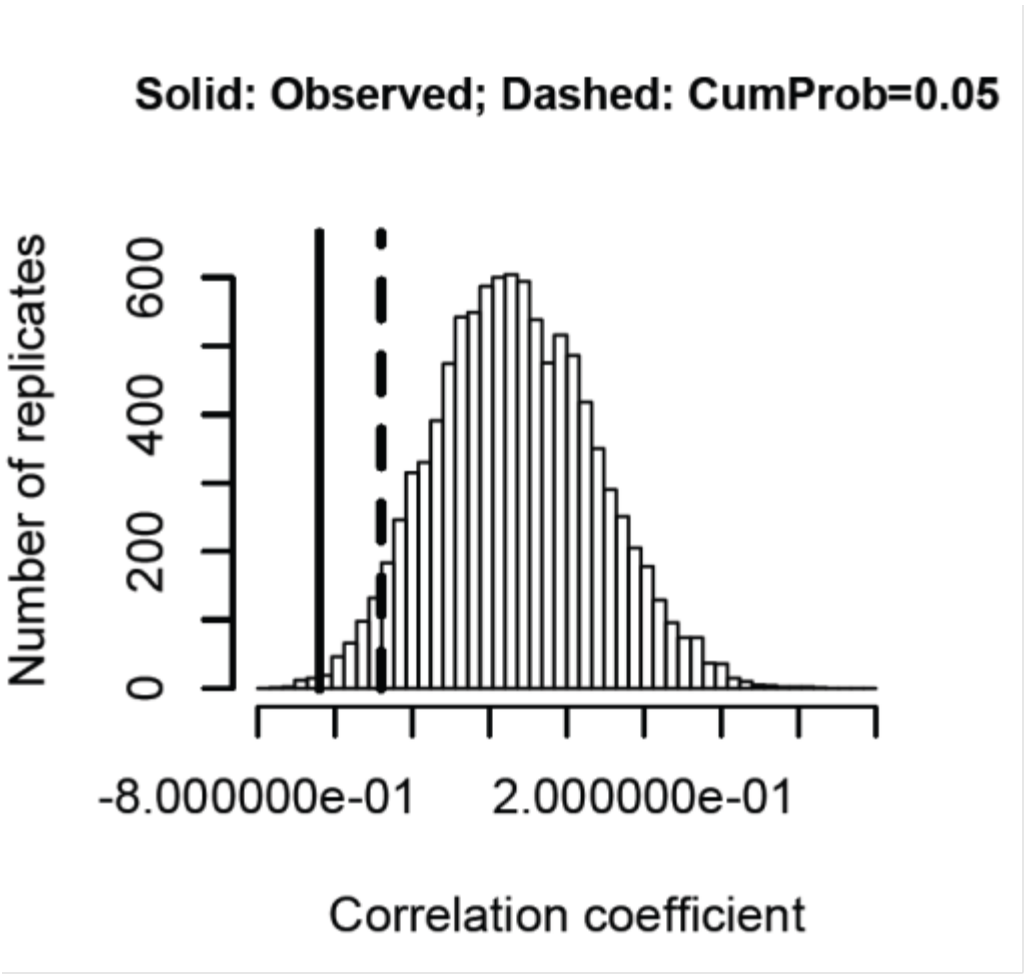


Figure 35. Permutation test results (N= 10,000) showing that the observed correlation coe.fficient (solid line) is significantly more negative than the lower 5% tail (dotted line) of the calculated distribution of possible correlation coefficients that could be calculated from the dataset (P=0.0049).

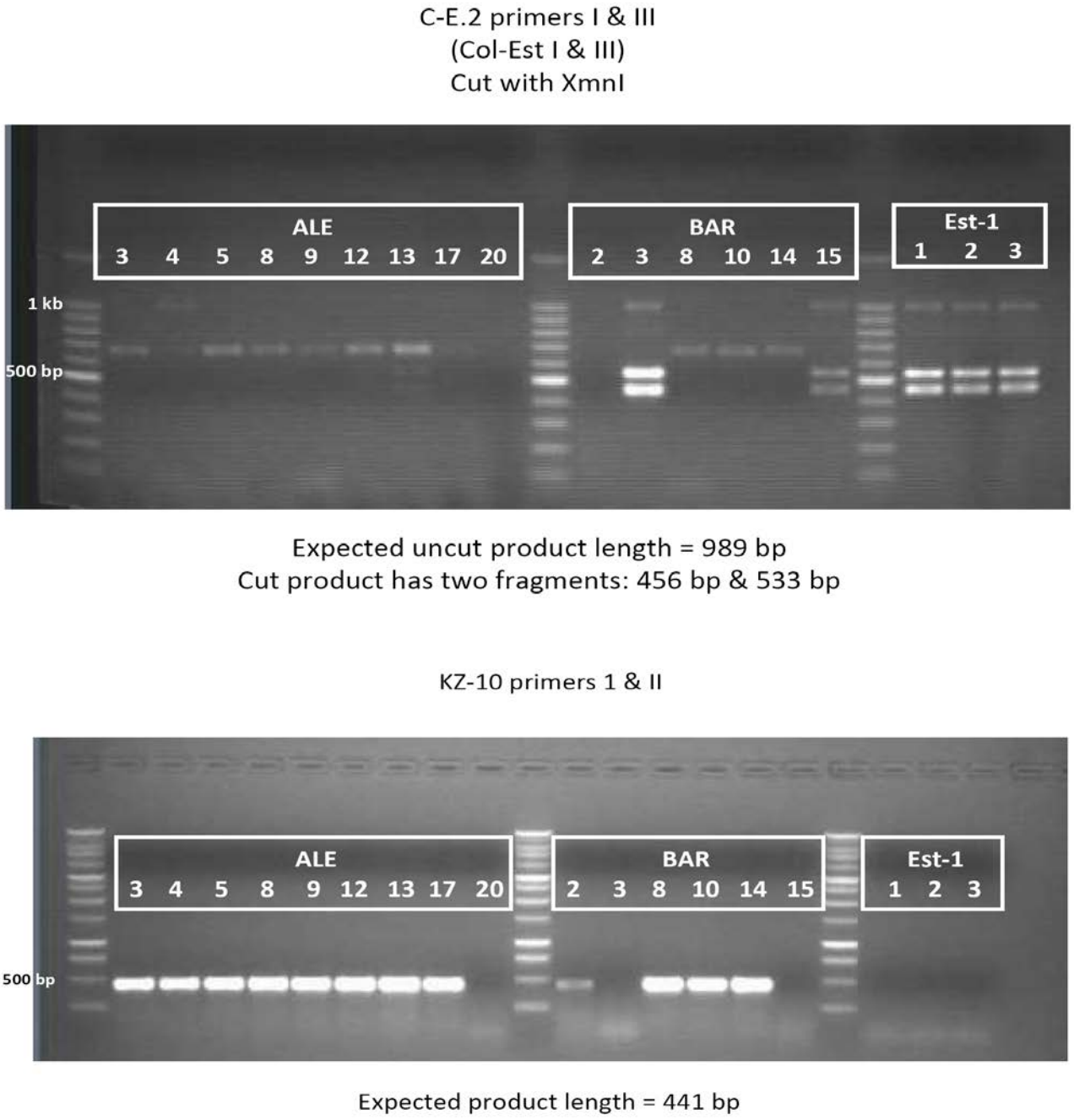


Figure 36. PCR amplification of Col-0, Est-1, and KZ-10 alleles at the ACD6 locus for the ALE (1225m elevation) and BAR (429m elevation) populations, with the Est-1 genotype as a positive control. The Est-1 sequence amplified at the ACD6 locus contains a restriction site that is cut by the XmnI restriction enzyme, whereas the Col-0 sequence does not have this restriction site (Todesco et al. 2010).

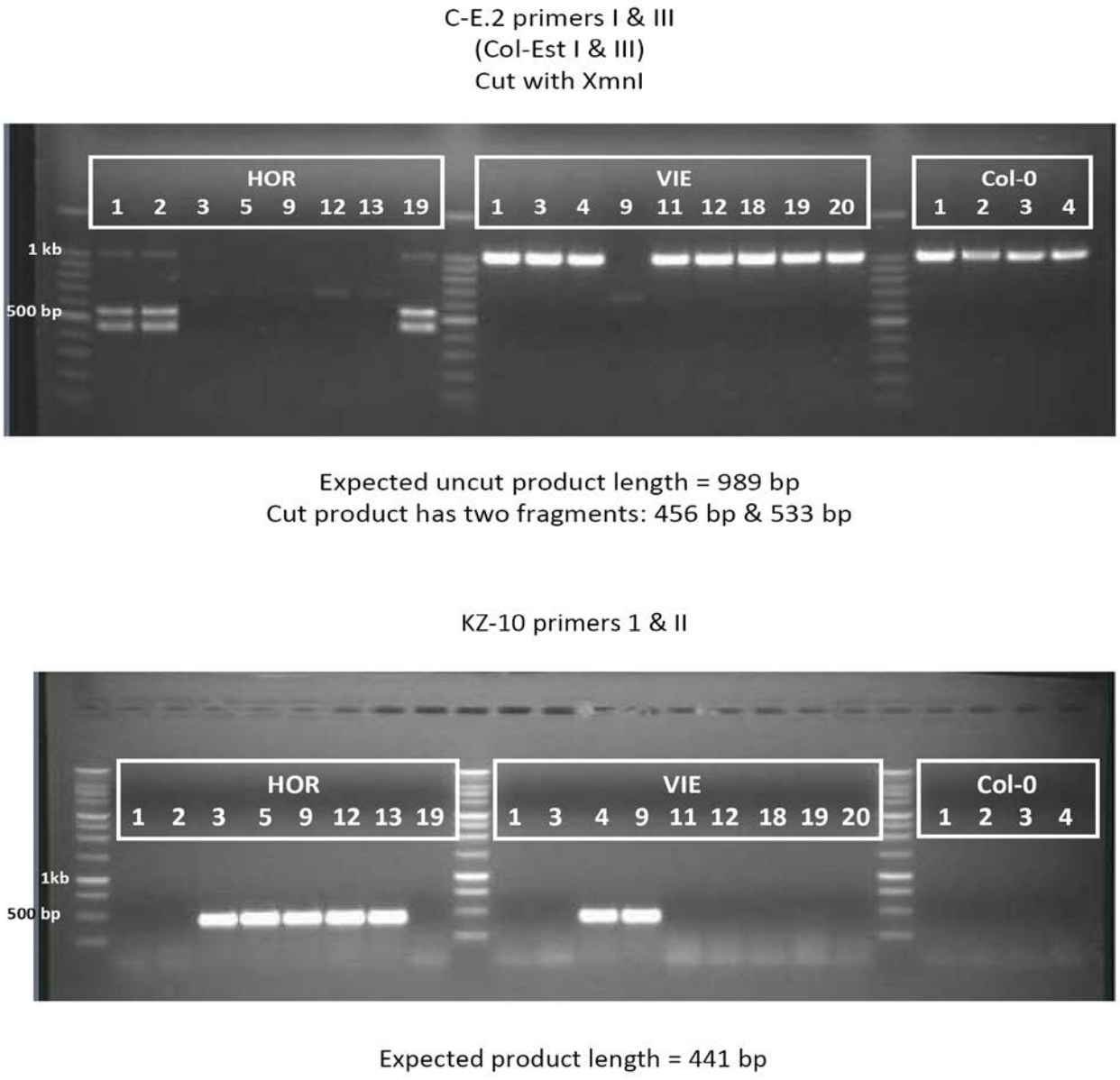


Figure 37. PCR amplification of Col-0, Est-1, and KZ-10 alleles at the ACD6 locus for the HOR (431m elevation) and VIE (1600m elevation) populations, with the Col-0 genotype as a positive control. The Est-1 sequence amplified at the ACD6 locus contains a restriction site that is cut by the XmnI restriction enzyme, whereas the Col-0 sequence does not have this restriction site (Todesco et al. 2010).

APPENDIX C

SUPPLEMENTARY MATERIALS FOR CHAPTER FOUR

Table 24. Significance values for tests of within population variation in seedling survival, post-stress root growth and Hsp101 expression. Each treatment was tested in a separate analysis.

Treatment	Seedling survival	Post-stress root growth	Hsp101 expression
T42	0.38	<.0001	0.16
.\T42	0.001	<.0001	0.07
BT45	0.02	<.0001	0.54
T45	0.008	0.0001	0.59

CT42: 42°C for 3hrs; CT45: 45°C for 3hrs; AT42: 3hrs at 38°C, recovery at 22°C for 3hrs, then 3hrs at 42°C; AT45: 3hrs at 38°C, recovery at 22°C for 3hrs, then 3hrs at 45°C.

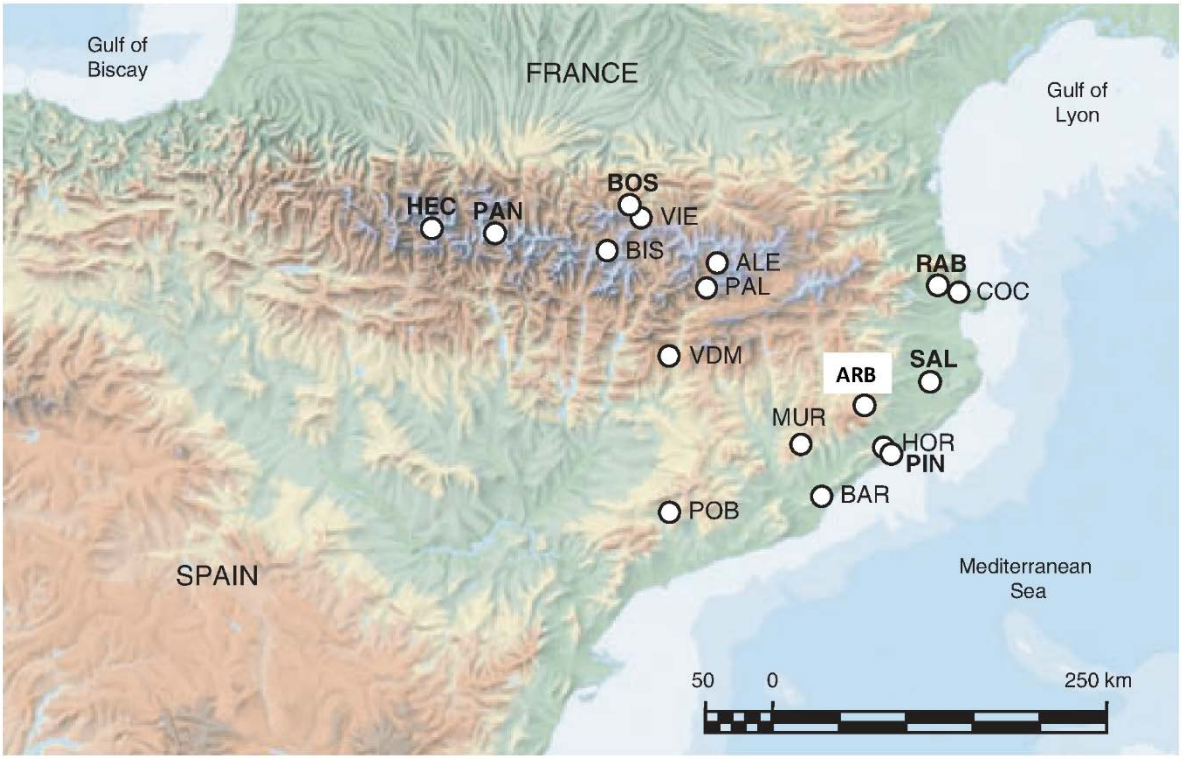


Figure 38. Geographic location of the 16 populations used in this study (From Fig.1, Montesinos-Navarro et al. 2011).

APPENDIX D

SUPPLEMENTARY MATERIALS FOR CHAPTER FIVE

Table 25. ANOVA table for each variable measured in this study.

Variables	Elevation group	Heat treatment	Elevation group * Heat treatment	Population (Elevation group)
Total fruit length	<0.0001	0.0007	NS	<0.0001
Basal branches number	<0.0001	0.005	NS	<0.0001
Reproductive length	<0.0001	0.01	NS	<0.0001
Root dry mass	<0.0001	0.001	NS	0.004
Rosette Temperature (DeltaT)	NS	<0.0001	0.02	0.007
Rosette Angle	0.0005	NS	NS	0.02
Transpiration Rate	<0.0001	<0.0001	0.007	<0.0001
Photosynthetic Rate	<0.0001	0.007	0.003	0.02
Hsp101 accumulation	0.05	<0.0001	NS	NS
Free salicylic acid accumulation (log)	0.0001	NS	NS	0.003
Total salicylic acid accumulation (log)	0.07	NS	0.03	0.002

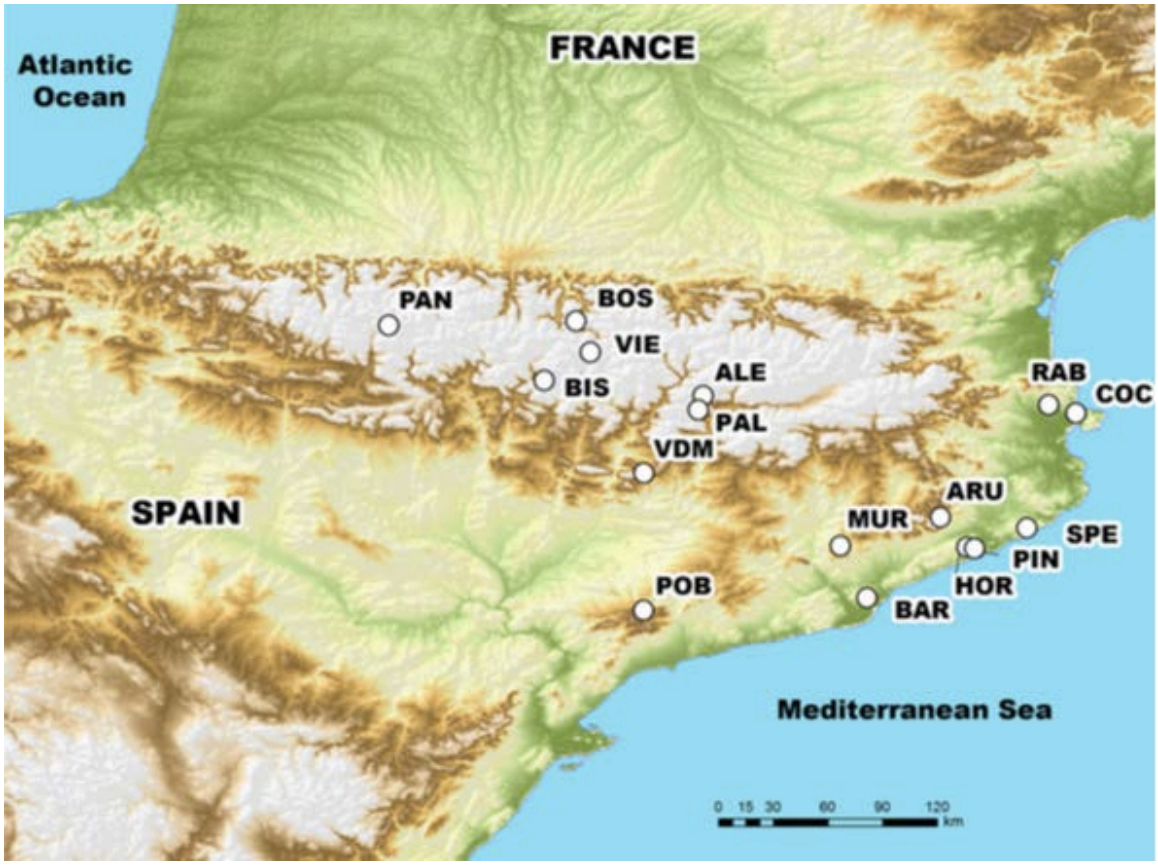


Figure 39. Geographic location of the 8 populations (adapted from Fig.1 in Wolfe and Tonsor 2014, New Phytologist). Map showed all 16 Arabidopsis thaliana populations in northeastern Spain. The eight populations used in this study were highlighted with black dots.

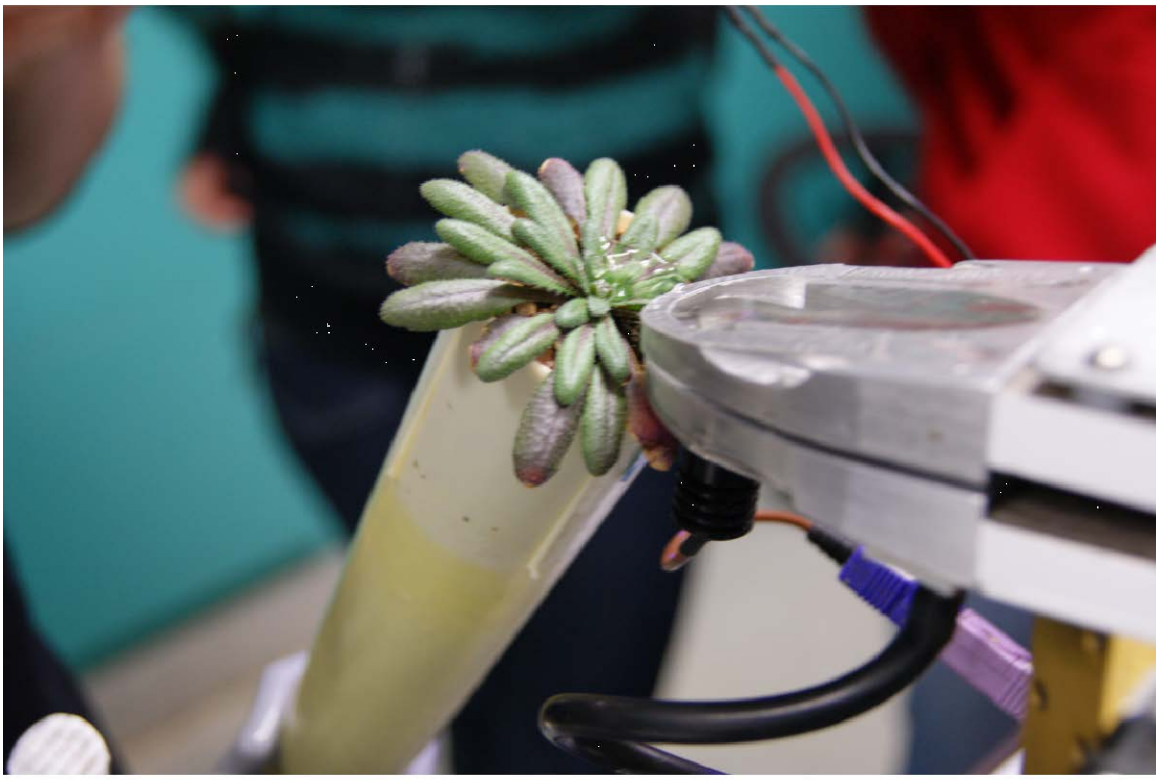


Figure 40. Using single leaf cuvette to quantify photosynthetic rate and transpiration rate. Plants shown were at the pre-bolting stage. Here we only use this photo to show the equipment used for quantification. We do not have a figure to show the measurement at the bolting stage.

Figure 41. Heat stress caused more inflorescences to be induced. Red arrows showed the regions where the stems were damaged, and green arrows showed new branches initiated after heat stress. Figure shows both the whole reproductive part and the detailed heat stress disruption of shoot apices.

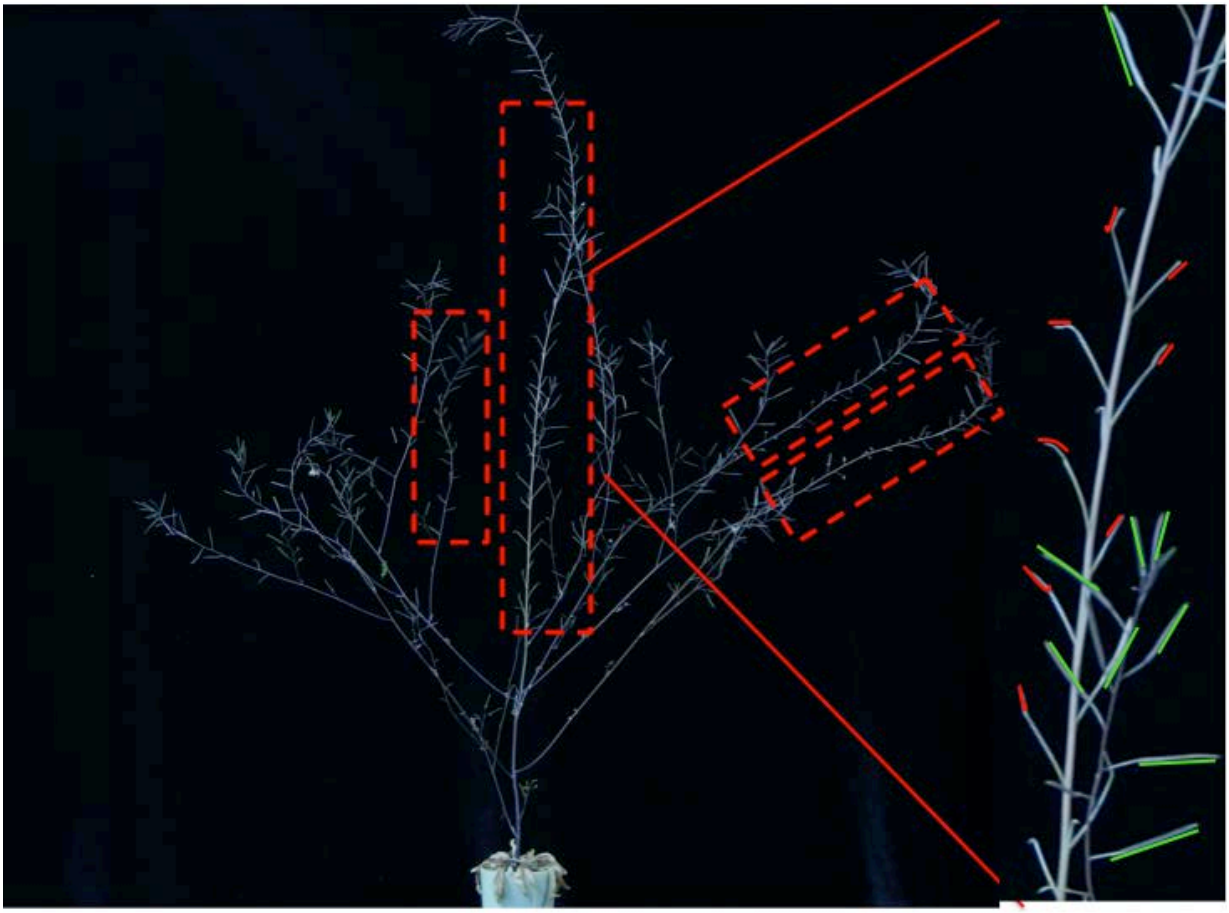


Figure 42. Heat stress reduced fruit quality. Damage occurred in the middle of the main stem at a time when that stem segment was near the stem apex. After heat stress, the main stem growth recovered, but the fruits did not succeed to mature, possibly because of pollen inviability. Dissection on fruits of this kind showed that they did not produce viable seeds. Figure shows both the damage on the whole reproductive part and a enlarged view of section of the stem containing heat-damaged fruit. In the enlargment, the short red lines mark damaged fruits and the short green lines mark normally developed fruit. The damaged fruits are much smaller compared to a normal fruit. The smaller dashed rectangles highlight additional stems with similar damage.

APPENDIX E

SUPPLEMENTARY MATERIALS FOR CHAPTER SIX

Table 26. Alignment summary of RNA-seq data for the 24 samples to *Arabidopsis thaliana* (Tair 10) transcriptome with Tophat2.

Sample	Elevation group	Treatment	Number Raw Reads	Number Mapped Reads	% of total mapped
HOR7	Low	22°C	27736919	24308845	87.6
HOR7	Low	45°C	27735098	24823054	89.5
HOR7	Low	38-45°C	34285786	26806810	78.2
PIN9	Low	22°C	35250112	31290257	88.8
PIN9	Low	45°C	27414384	24493775	89.3
PIN9	Low	38-45°C	22861792	8279162	80.0
RAB4	Low	22°C	24956107	22166537	88.8
RAB4	Low	45°C	24910779	22711036	91.2
RAB4	Low	38-45°C	27280627	21763377	79.8
SPE6	Low	22°C	26441088	23290731	88.1
SPE6	Low	45°C	27479845	24828659	90.4
SPE6	Low	38-45°C	35813970	28071470	78.4
BIS8	High	22°C	24102301	21236376	88.1
BIS8	High	45°C	27300909	24737693	90.6
BIS8	High	38-45°C	31933204	25013914	78.3
PAL6	High	22°C	26127566	23085888	88.4
PAL6	High	45°C	25747254	23168349	90.0
PAL6	High	38-45°C	32030973	25127204	78.4

PAN8	High	22°C	26737620	23502729	87.9
PAN8	High	45°C	26428191	23708370	89.7
PAN8	High	38-45°C	30941863	24415423	78.9
VIE4	High	22°C	30350162	26693836	88.0
VIE4	High	45°C	26457362	23935965	90.5
VIE4	High	38-45°C	27106330	21442774	79.1

Note: The summary was based on alignment results after trimming out the first and last 15bp for each 100bp reads.

Table 27. Currently known heat stress related genes investigated in this study.

Gene family	Gene description
Hsp-Hsf pathway:	
Hsfs	Heat shock factors
Hsps	Heat shock proteins
ROS pathway:	
Zat family (Zat7, Zat10, Zat12)	Zinc transporter family protein, responds to diversified stress, including heat stress.
WRKY family (WRKY25)	WRKY transcription factor, involve in response to heat and other stress.
AP2/ERF family (DREB2A, DREB2B, ETR1, EIN2)	Plant specific transcription factor, activates the expression of abiotic stress-responsive genes.
MBF1c	Highly conserved transcriptional co-activator, involve in thermotolerance.
RBoh	ROS (reactive oxygen species) signal amplifier.
CBK3	Important component of Ca2+ -regulated heat stress signal transduction pathway, downstream of CaM, which regulates Hsps expression.
BOB1 (BOBBER1)	A small Hsp with a thermotolerance role at high temperature.
ABA signaling (ABI1, ABI2)	Reduced survival after heat stress in these mutants, however the accumulation of Hsps was not affected.
NDH1	High heat-inducible and provide protection against photo-oxidation.
PP7	Encodes a nuclear localized serine/threonine phosphatase that appears to be regulated by redox activity and is a positive regulator of cryptochrome mediated blue light signalling.
BI1	BAX inhibitor 1, Functions as an attenuator of biotic and abiotic types of cell death.
UVH6	A negative regulator of the common stress response induced by UV damage and heat.
VPS53	Involved in vesicle trafficking, heat stress sensitive gene.
CTL1	A chitinase-like protein, required for acquired thermotolerance, salt stress and development.
FtsH11	Chloroplastic FstH11 protease, associated with strongly reduced photosynthetic capacity after heat stress.
DGD1	Digalactosyldiacylglycerol synthase 1, associated with strongly reduced photosynthetic capacity after heat stress.
TU8/TFL2	Terminal Flower 2, which shows Hsp90 reduction in the tu8 mutant.

Note: the above gene list is summarized from three review papers (Kotak et al. 2007, Ahuja et al. 2010, Qu et al. 2013). Gene functions were further confirmed from the NCBI website.

APPENDIX F

SUPPLEMENTARY MATERIALS FOR CHAPTER SEVEN

Table 28. The candidate genes involved in adaptation to very high elevation condition when compared Very high vs. High/middle and Very high vs. low, separately.

Gene ID	Gene Name	Gene description
Very high vs. High/middle		
AT1G64380.1	ERF061	Ethylene-responsive transcription factor ERF061.
AT2G01320.3	ABCG7	ABC transporter G family member 7.
AT2G01330.2		Nucleotide binding.
AT2G18790.1	PHYB	Phytochrome B.
AT5G32590.1		Myosin heavy chain-related.
AT5G32597.1		Unknown protein; Has 30201 Blast hits to 17322 proteins in 780 species.
AT5G33280.1	CBSCLC6	Putative chloride channel-like protein CLC-g.
AT5G33390.1		Glycine-rich protein.
Very high vs. Low		
AT1G20100.1		Unknown protein; BEST Arabidopsis thaliana protein match is: unknown protein (TAIR:AT1G75860.1).
AT1G20110.1		RING/FYVE/PHD zinc finger superfamily protein.
AT1G64380.1	ERF061	Ethylene-responsive transcription factor ERF061.
AT4G28680.4	TYRDC	Probable tyrosine decarboxylase 2.
AT4G28690.1		BEST Arabidopsis thaliana protein match is: RPM1 interacting protein 13 (TAIR:AT2G20310.1).
AT4G28700.1	AMT1-4	Ammonium transporter 1 member 4.
AT5G35603.2		Protein of unknown function (DUF3287).
AT5G25604.1		BEST Arabidopsis thaliana protein match is: myosin heavy chain-related (TAIR: AT5G32590.1).
AT5G49150.1	GEX2	Protein GAMETE EXPRESSED 2.

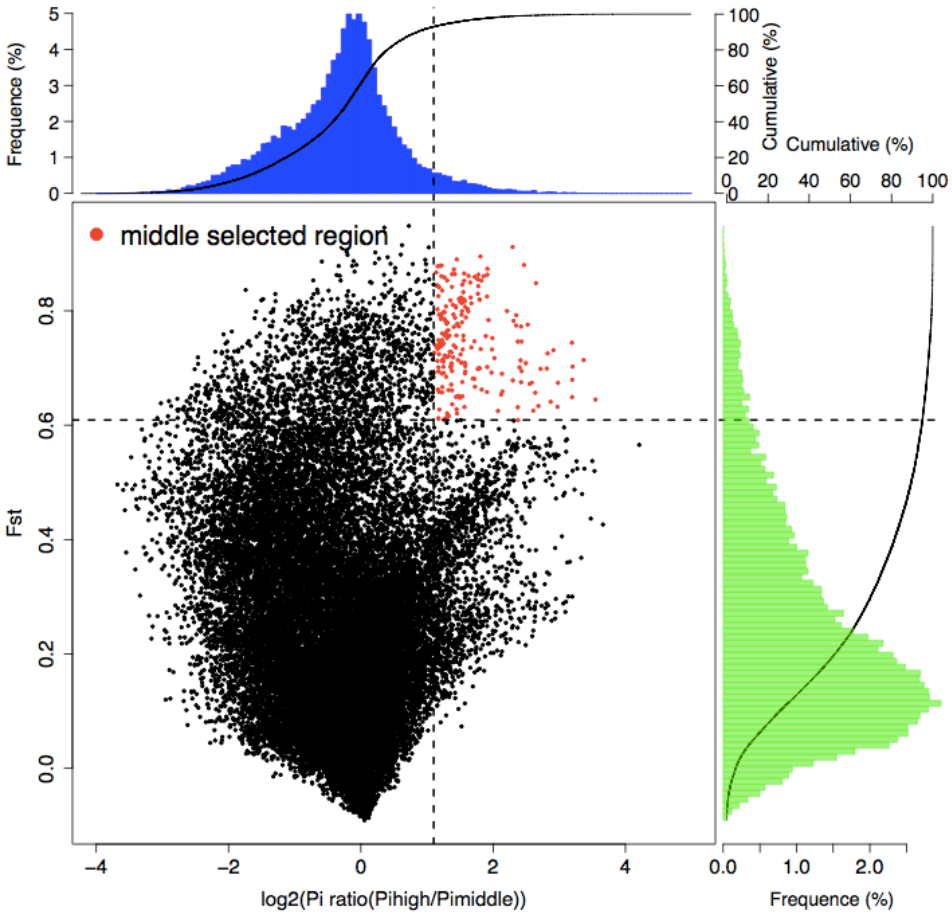


Figure 43. Selective sweep result of high/middle elevation group comparing with very high elevation group based on Fst and Pi, as an example to show selective sweep analysis. This analysis is calculated in 5kb windows sliding in 1kb steps. Data points marked with red are suggested to be selective regions (corresponding to the 5% values of *Fst* and Pi ratio). Data points located to the left and right of the left and right vertical dashed lines, respectively (corresponding to the 5% left and right tails of the empirical ratio distribution, where the ratios are 0.57 and 1.10, respectively), and above the horizontal dashed line (the 5% right tail of the empirical F_{ST} distribution, where F_{ST} is 0.361) were identified as selected regions for high/middle elevation (blue points) and very high elevation (green points), respectively.

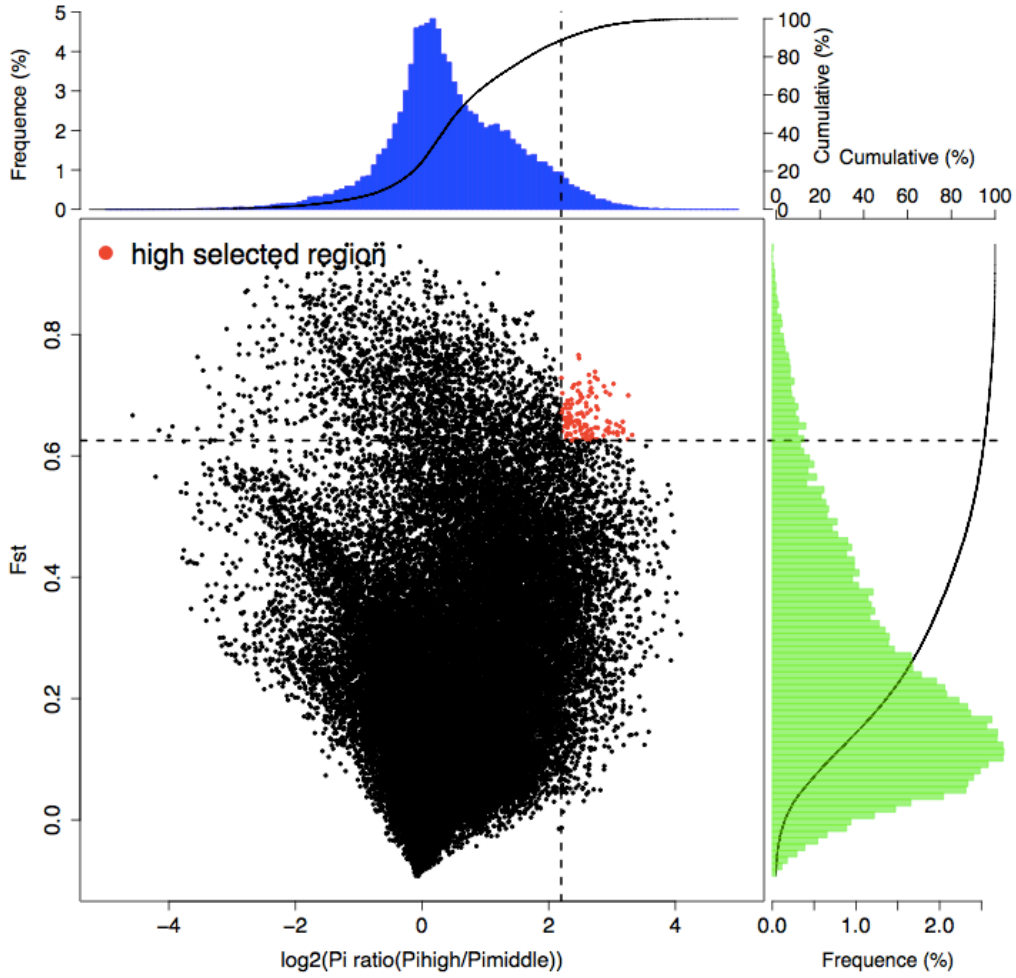


Figure 44. FigSelective sweep result of very high elevation group comparing with high/middle elevation group based on Fst and Pi, as an example to show selective sweep analysis. This analysis is calculated in 5kb windows sliding in 1kb steps. Data points marked with red are suggested to be selective regions (corresponding to the 5% values of *Fst* and Pi ratio). Data points located to the left and right of the left and right vertical dashed lines, respectively (corresponding to the 5% left and right tails of the empirical ratio distribution, where the ratios are 0.57 and 1.10, respectively), and above the horizontal dashed line (the 5% right tail of the empirical F_{ST} distribution, where F_{ST} is 0.361) were identified as selected regions for very high elevation (blue points) and high/middle elevation (green points), respectively.

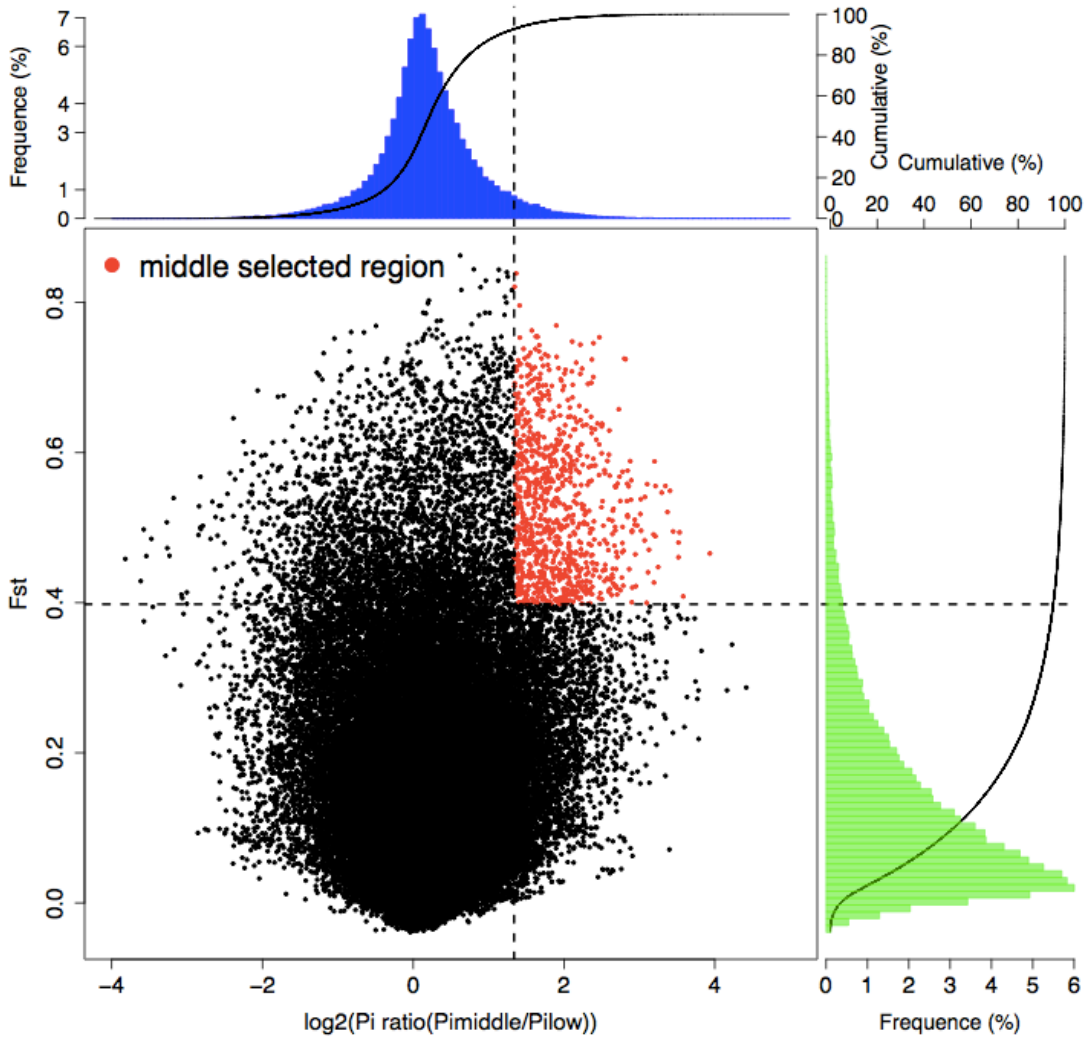


Figure 45. Selective sweep result of high/middle elevation group comparing with low elevation group based on Fst and Pi, as an example to show selective sweep analysis. This analysis is calculated in 5kb windows sliding in 1kb steps. Data points marked with red are suggested to be selective regions (corresponding to the 5% values of *Fst* and Pi ratio). Data points located to the left and right of the left and right vertical dashed lines, respectively (corresponding to the 5% left and right tails of the empirical ratio distribution, where the ratios are 0.57 and 1.10, respectively), and above the horizontal dashed line (the 5% right tail of the empirical F_{ST} distribution, where F_{ST} is 0.361) were identified as selected regions for high/middle elevation (blue points) and low elevation (green points), respectively.

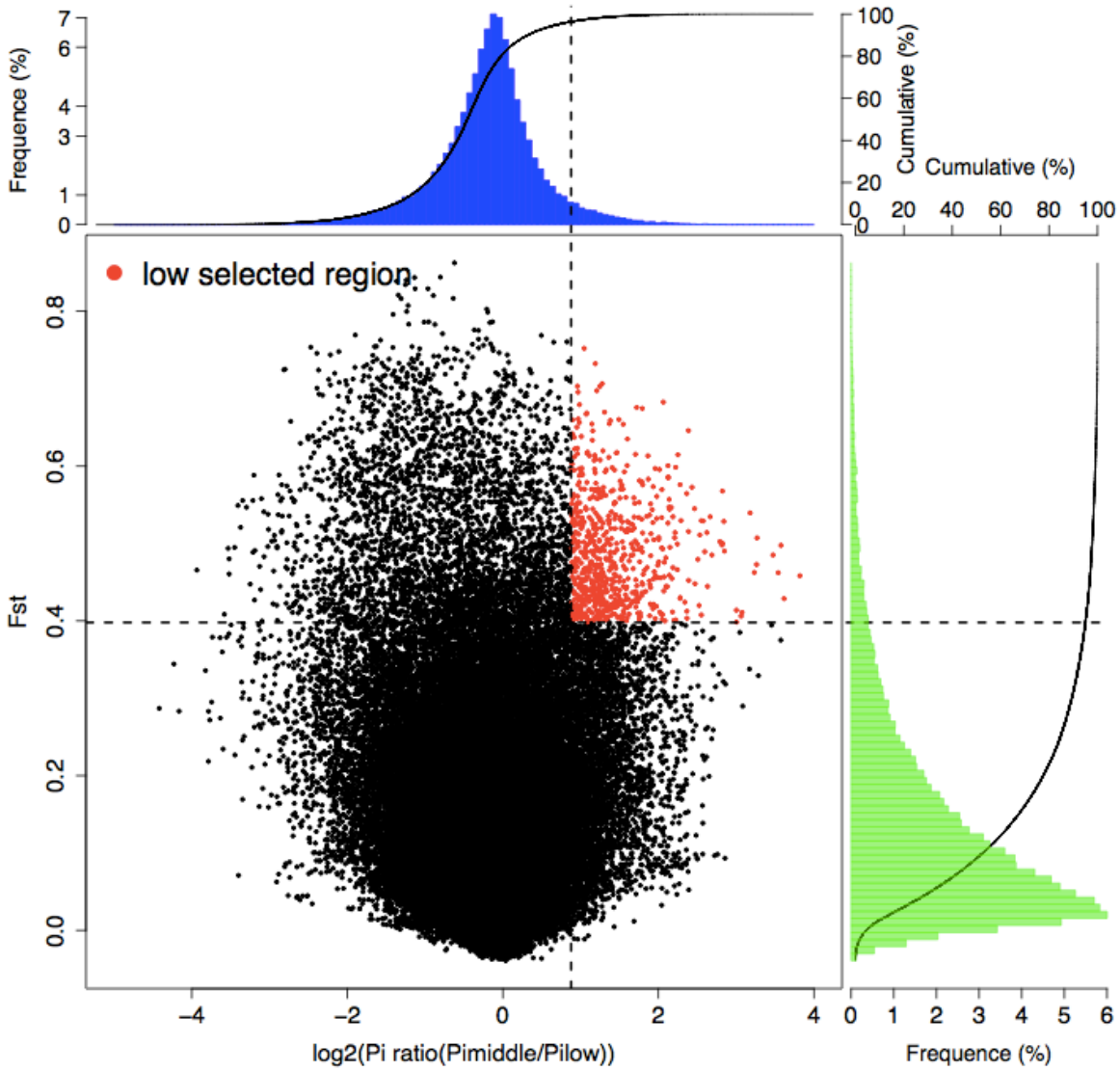


Figure 46. Selective sweep result of low elevation group comparing with high/middle elevation group based on Fst and Pi, as an example to show selective sweep analysis. This analysis is calculated in 5kb windows sliding in 1kb steps. Data points marked with red are suggested to be selective regions (corresponding to the 5% values of *Fst* and Pi ratio). Data points located to the left and right of the left and right vertical dashed lines, respectively (corresponding to the 5% left and right tails of the empirical ratio distribution, where the ratios are 0.57 and 1.10, respectively), and above the horizontal dashed line (the 5% right tail of the empirical F_{ST} distribution, where F_{ST} is 0.361) were identified as selected regions for low elevation (blue points) and high/middle elevation (green points), respectively.

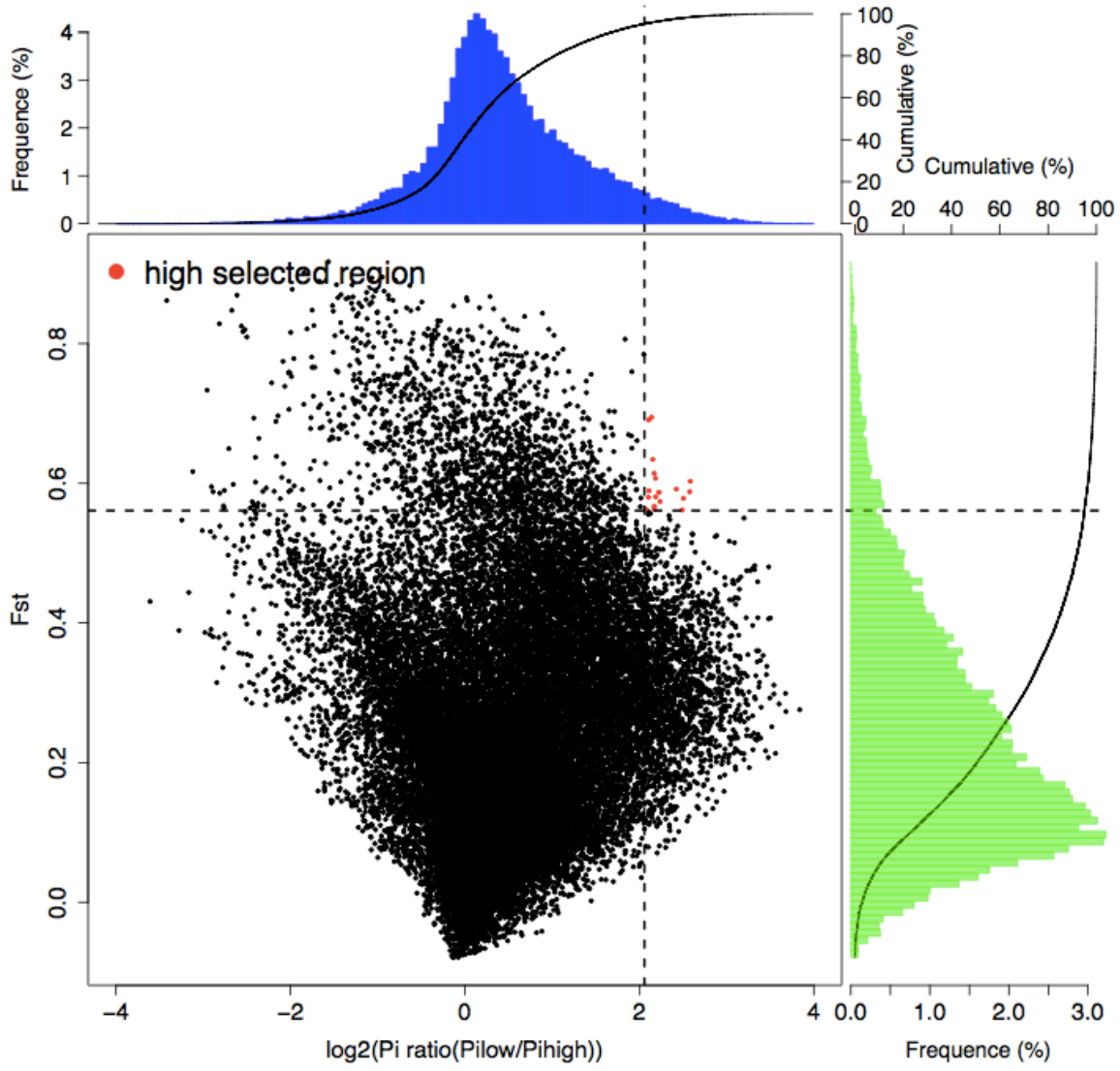


Figure 47. Selective sweep result of very high elevation group comparing with low elevation group based on Fst and Pi, as an example to show selective sweep analysis. This analysis is calculated in 5kb windows sliding in 1kb steps. Data points marked with red are suggested to be selective regions (corresponding to the 5% values of *Fst* and Pi ratio). Data points located to the left and right of the left and right vertical dashed lines, respectively (corresponding to the 5% left and right tails of the empirical ratio distribution, where the ratios are 0.57 and 1.10, respectively), and above the horizontal dashed line (the 5% right tail of the empirical F_{ST} distribution, where F_{ST} is 0.361) were identified as selected regions for very high elevation (blue points) and low elevation (green points), respectively.

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